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(71) **Demandeurs/Applicants:**
CELLULAR DYNAMICS INTERNATIONAL, INC., US;
FUJIFILM HOLDINGS AMERICA CORPORATION, US

(72) **Inventeurs/Inventors:**
RAJESH, DEEPIKA, US;
MUNN, CHRISTIE, US;
BURTON, SARAH, US;
DONEGAN, MADELYN ELISE, US;
OSHIMA, MAKIKO, US;
CARLSON, COBY, US;
...

(74) **Agent:** BERESKIN & PARR LLP/S.E.N.C.R.L.,S.R.L.

(54) Titre : COMPOSITIONS DE CELLULES DERIVEES DE CELLULES SOUCHES PLURIPOTENTES INDUITES ET LEURS PROCEDES D'UTILISATION

(54) Title: COMPOSITIONS OF INDUCED PLURIPOTENT STEM CELL-DERIVED CELLS AND METHODS OF USE THEREOF

(57) **Abrégé/Abstract:**

The present disclosure provides multicellular culture models for the study of neuroinflammation, such as to identify novel targets, biomarkers, and therapeutic agents for the diagnosis, prognosis, and treatment of neurodegenerative diseases. Further provided herein are assays for studying neuroinflammation using the present cell culture models.

(72) **Inventeurs(suite)/Inventors(continued):** LIVINGSTON, MEGAN, US; TOMOTOSHI, KIMIHIKO, US; FATHI, ALI, US; LIU, JING, US; POON, WAYNE, US; HILCOVE, SIMONE, US; BAKSHY, KIRANMAYEE, US

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Abstract:

The present disclosure provides multicellular culture models for the study of neuroinflammation, such as to identify novel targets, biomarkers, and therapeutic agents for the diagnosis, prognosis, and treatment of neurodegenerative diseases. Further provided herein are assays for studying neuroinflammation using the present cell culture models.

DESCRIPTION

COMPOSITIONS OF INDUCED PLURIPOTENT STEM CELL-DERIVED CELLS AND METHODS OF USE THEREOF

PRIORITY CLAIM

[0001] This application claims benefit of priority to U.S. Provisional Application Serial No. 63/242,900 filed September 10, 2021, the entire contents of which are hereby incorporated by reference.

INCORPORATION OF SEQUENCE LISTING

[0002] This application contains a Sequence Listing XML, which has been submitted electronically and is hereby incorporated by reference in its entirety. Said XML Sequence Listing, created on September 12, 2022, is named CDINP0110WO.xml and is 2,828 bytes in size.

BACKGROUND

1. Field

[0003] The present invention relates generally to the field of molecular biology and medicine. More particularly, it concerns compositions of cells differentiated form induced pluripotent stem cells and methods of use thereof.

2. Description of Related Art

[0004] Immune function and in particular, tissue resident macrophages play an integral role in disease pathogenesis. For example, the neuro-immune axis and microglia, the brain resident macrophage, play an essential role in neurodegenerative disease pathobiology including Alzheimer's disease, which is supported by both Genome-wide Association Studies and Omics studies. In addition, tissue resident macrophages play important roles in the pathogenesis of NASH (Kupffer cells), AMD (sub-retinal microglia), asthma, COPD (lung alveolar macrophages), and HIV. Numerous studies have also identified lipid regulatory dysfunction contributing to retinal microglia drusen formation, atherosclerotic plaque formation (peripheral macrophages), pulmonary foam cells, and brain AD neuropathology. Understanding how lipid dysfunction of tissue resident macrophages affects homeostatic function can serve as a therapeutic avenue for a multitude of chronic diseases with an inflammation etiology.

SUMMARY

[0005] In some embodiments, the present disclosure provides cell cultures comprising induced pluripotent stem cell (iPSC)-derived microglia, astrocytes, and/or neurons in media. Further provided herein are methods of producing the cell culture and methods of use thereof.

[0006] In certain aspects, the culture comprises iPSC-derived microglia, astrocytes, and neurons. In specific aspects, the cell culture is further defined as a triculture.

[0007] In some aspects, the neurons are excitatory neurons or inhibitory neurons. In particular aspects, the neurons are gabaergic neurons, dopaminergic neurons, or glutamatergic neurons.

[0008] In certain aspects, the microglia are derived from isogenically engineered iPSC lines. In particular aspects, the microglia, astrocytes and neurons are isogenic. In some aspects, the microglia are at least 90% (e.g., 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) positive for TREM2, P2RY12, TMEM119, IBA-1, and/or CX3CR1. In specific aspects, the microglia are mature microglia. In certain aspects, the astrocytes are positive for S100 beta, GFAP, and CD44. In some aspects, the neurons are positive for at least two (e.g., 3, 4, 5, 6, 7, 8, 9, 10 or more) of the markers selected from the group consisting of SCL1, BCL11B, Calb2, CD24, CDH1, CUX1 Cux2, DCX, DLG4, Dlx, Dlx2, Emx1, Emx2, eomes, ETV1, FOXG1, FOXP2, Fut4, GABRA2, GAD1, GAD2, GAPDH, GFAP, GRIN2B, HoxB4, HTR2C, ISL1, ITGB1, LHX2,Neurog1, NKX2-1, Nos1, NPY, NR4A2, PAX6, POU3F2, PVALB, RELN , SATB2, SLC17A6, SLC17A7, SLC17A8, SLC32A1, SOX1, Sox10, SST, SYN1, and Tbr1.

[0009] In certain aspects, the microglia are derived from donors expressing disease-associated SNPs. In some aspects, the microglia are generated from disease associated iPSC donors with TREM2, APOE, CD33, BIN, ABCA7, SNPS or genotypes associated with neurodegeneration. In some aspects, the microglia comprise a disruption in TREM2, Methyl-CpG Binding Protein 2 (MeCP2), and/or Alpha-synuclein (SCNA). In particular aspects, the microglia comprise a disruption in TREM2. For example, the disruption in TREM2 comprises a TAL nuclease-mediated disruption at amino acid 58 in exon 2 of TREM2. In some aspects, the microglia comprise a heterozygous disruption of TREM2. In other aspects, the microglia comprise a homozygous disruption of TREM2.

[0010] In some aspects, the cell culture is a two-dimensional (2D) culture. In certain aspects, the media further comprises IL-34 and M-CSF, or analogs or mimetics thereof. For example, the media further comprises IL-34 at a concentration of 50-200 ng/mL (e.g., 50, 75, 100, 150, 175, or 200 ng/mL, particularly 100 ng/mL) and M-CSF at a concentration of 10-50 ng/mL (e.g., 10, 25, 30, 40, or 50 ng/mL, particularly 25 ng/mL). In additional aspects, the media further comprises TGFβ or analogs or mimetics thereof, such as at a concentration of 10-100 ng/mL (e.g., 10, 25, 30, 40, 50, 75, 80, 90, or 100 ng/mL, particularly 50 ng/mL).

[0011] In some aspects, the cells are cultured or positioned on a cell surface. In certain aspects, the cells are cultured on a surface coated with polyethyleneimine (PEI). In some aspects, the cells are cultured on a surface coated with an extracellular matrix protein. In particular aspects, the extracellular matrix is basement membrane extract (BME) purified from murine Engelbreth-Holm-Swarm tumor. In some aspects, the extracellular matrix protein is MATRIGEL®, GELTREX™, collagen, or laminin. For example, the extracellular matrix protein is GELTREX™ or laminin.

[0012] In some aspects, the cell culture is a three-dimensional (3D) culture. In specific aspects, the 3D culture is a brain organoid culture. In some aspects, the 3D culture comprises a functional neuron network. In particular aspects, the functional neuron network comprises calcium oscillations.

[0013] In some aspects, the cultures comprises the microglia and astrocytes present in a ratio of about 3:1 to 1:3, particularly about 2:1 to 1:2, such as about 1:1. In certain aspects, the microglia and astrocytes were seeded in the culture at a ratio of 3:1 to 1:3, particularly about 2:1 to 1:2, such as about 1:1. In certain aspects, the culture comprises the microglia, astrocytes, and neurons in a ratio of about 1:1:5 to 1:1:10, such as 1:1:6, 1:1:7, 1:1:8, 1:1:9, or 1:1:10. In certain aspects, the culture comprises the microglia, astrocytes, and neurons that were seeded in a ratio of about 1:1:5 to 1:1:10, such as 1:1:5, 1:1:6, 1:1:7, 1:1:8, 1:1:9, or 1:1:10, particularly about 1:1:5. In some aspects, the microglia, astrocytes, and neurons are in a ratio of about 3:1:5 to 1:3:10, such as about 3:1:5, 3:1:6, 3:1:7, 3:1:8, 3:1:9, 3:1:10, 3:2:5, 3:2:6, 3:2:7, 3:2:8, 3:2:9, 3:2:10; 1:3:5, 1:3:6, 1:3:7, 1:3:8, 1:3:9, 1:3:10, 2:3:5, 2:3:6, 2:3:7, 2:3:8, 2:3:9, 2:3:10, 2:1:5: 2:1:6, 2:1:6, 2:1:7, 2:1:8, 2:1:9, 2:1:10, 1:2:5, 1:2:6, 1:2:7, 1:2:8, 1:2:9, or 1:2:10 or any range derivable within. In some aspects, the microglia, astrocytes, and neurons were seeded in a ratio of about 3:1:5 to 1:3:10, such as about 3:1:5, 3:1:6, 3:1:7, 3:1:8, 3:1:9, 3:1:10, 3:2:5, 3:2:6, 3:2:7, 3:2:8, 3:2:9, 3:2:10; 1:3:5, 1:3:6, 1:3:7, 1:3:8, 1:3:9, 1:3:10, 2:3:5, 2:3:6, 2:3:7, 2:3:8, 2:3:9, 2:3:10, 2:1:5: 2:1:6, 2:1:6, 2:1:7, 2:1:8, 2:1:9, 2:1:10, 1:2:5, 1:2:6, 1:2:7, 1:2:8, 1:2:9, or 1:2:10 or any range derivable within. In some aspects, the culture comprises microglia that were seeded at a cell density of 5,000 cells/well to 7,500 cells/well. In certain aspects, the culture comprises neurons that were seeded at a cell density of 40,000 cells/well to 50,000 cells/well. In some aspects, the culture comprises astrocyte that were seeded at a cell density of 8,000 cells/well to 10,000 cells/well. the culture comprises microglia at a cell density of 15,000 cells/cm^2 to 25,000 cells/cm^2, such as 15,625 cells/cm^2 to 23,438 cells/cm^2. In some aspects, the culture comprises neurons at a cell density of 125,000 cells/cm^2 to 160,000 cells/cm^2, such as 125,000 cells/cm^2 to 156,250 cells/cm^2. In certain aspects, the culture comprises astrocyte at a cell density of 25,000 cells/cm^2 to 35,000 cells/cm^2, such as 25,000 cells/cm^2 to 31,250 cells/cm^2.

[0014] In certain aspects, the microglia, astrocytes, and neurons have been in the culture for at least 10 day, such as at least 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more days. In particular aspects, the microglia, astrocytes, and neurons have been in culture at least 14 days. In some aspects, the microglia, astrocyte, and neurons have been in culture at least 14 days and are present at a ratio of 2:6:1. In some aspects, the microglia, astrocyte, and neurons have been in culture at least 14 days and are present at a ratio of about 15:1:1 to 1:15:30, such as about 10:1:1 to 1:10:20, about 8:1:1 to 1:8:20, about 5:1:1 to 1:5:20, or about 3:1:1 to 1:3:20.

[0015] In some aspects, the iPSCs are human. In certain aspects, the culture is xeno-free, feeder-free, and/or conditioned-media free. In specific aspects, the media is defined media.

[0016] Another embodiment provides a method for culturing microglia and neurons. In some aspects, the microglia and neurons are isogenic. In some aspects, the neurons are GABAergic or glutamatergic neuron. In certain aspects, the co-culture of GABAergic or glutamatergic neurons and astrocytes results in enhanced phagocytic activity by the microglia compared to monoculture of microglia. In some aspects, the method further comprises detecting soluble TREM2. In some aspects, the astrocytes are isogenic

[0017] A further embodiment provides a cell culture comprising brain microvascular endothelial cells (BMECs), pericytes, and astrocytes in a sandwich format. In specific aspects, the culture is further defined as a blood brain barrier model.

[0018] In some aspects, the sandwich format comprises an extracellular matrix layer between two cell layers. In certain aspects, the extracellular matrix layer comprises at least two extracellular matrix proteins. For example, the at least two extracellular matrix proteins are collagen IV and fibronectin. In specific aspects, the sandwich format comprises BMECs on the apical side, the extracellular matrix layer in the middle, and astrocytes and pericytes on the basolateral side. In some aspects, the extracellular matrix layer further comprises gelatin.

[0019] In certain aspects, the sandwich format further comprises a permeable membrane insert. In some aspects, the permeable membrane insert is a polytetrafluoroethylene (PFTE), polycarbonate, or polyethylene terephthalate (PTE) insert. In some aspects, the permeable membrane insert is a PFTE insert. For example, the PFTE insert is a TRANSWELL™ insert. In some aspects, the permeable membrane insert is coated with human collagen IV and human fibronectin on the apical side. In some aspects, the permeable membrane insert is coated with human collagen IV at a concentration of 200-400 μg/mL, such as 200, 250, 300, 350, or 400 μg/mL, and human fibronectin at a concentration of 50-100 μg/mL, such as 50, 75, or 100 μg/mL. In certain aspects, the basolateral side of the permeable membrane insert is coated with gelatin, such as 0.01-0.2% gelatin, particularly about 0.1% gelatin.

[0020] In some aspects, the astrocytes and pericytes are present at an about 2:1 to 1:2 ratio, such as an about 2:1 ratio. In some aspects, the astrocytes and pericytes are seeded in the culture at an about 2:1 to 1:2 ratio, such as an about 2:1 ratio. In some aspects, the astrocytes and pericytes are on the basolateral side of the permeable membrane insert. In particular aspects, the BMECs are on the apical side of the permeable membrane insert.

[0021] In specific aspects, the BMECs are in media comprising EFRA2. In some aspects, the BMECs, astrocytes and pericytes are in media comprising a ROCK inhibitor, such as Y-27632.

[0022] In some aspects, the BMECs, astrocytes, and pericytes are present at a ratio of about 5:1:1 to 1:3:3, such as 4:1:2, 4:1:1, 4:2:1, or 2:1:1. In some aspects, the BMECs, astrocytes, and pericytes are seeded at a ratio of about 5:1:1 to 1:3:3, such as 4:1:2, 4:1:1, 4:2:1, or 2:1:1. In some aspects, the BMECs are seeded at a cell density of 1 x10^6 cells/cm^2 to 1.5x10^6 cells/cm^2, such as about 1.3x10^6. In specific aspects, the astrocytes are seeded at a cell density of 300,000 cells/cm^2 to 700,000 cells/cm^2, such as about 333,000 cells/cm^2. In certain aspects, the pericytes are seeded at a cell density of 300,000 cells/cm^2 to 700,000 cells/cm^2, such as about 666,000 cells/cm^2.

[0023] Another embodiment provides a method for screening a therapeutic compound for treating a neurodegenerative disease comprising contacting a test compound with a culture of the present embodiments and aspects thereof (e.g., cell culture comprising induced pluripotent stem cell (iPSC)-derived microglia, astrocytes, and/or neurons in media or cell culture comprising brain microvascular endothelial cells (BMECs), pericytes, and astrocytes in a sandwich format); and measuring the functional activity of the cells.

[0024] In some aspects, an increase in functional activity indicates the test compound is capable of treating a neurodegenerative disease. In certain aspects, measuring functional activity comprises measuring dendrite area (MAP2), synapse count, cell count, or axon area. In specific aspects, measuring the functional activity comprises detecting release of complement C3 from microglia. In certain aspects, a decrease in complement C3 released from the microglia indicates that the therapeutic compound is capable of treating a neurodegenerative disease.

[0025] In certain aspects, the method further comprises contacting the culture with LPS. In some aspects, measuring functional activity comprises measuring analytes released in the media with and without stimulation with LPS. For example, the analytes are M1 factors, such as TNF alpha, IL-6, CCL2, CCL3, CCL4, IL-1 beta, IL-12, IL-13, IL-8, Interferon gamma, IL1-Alpham FAS ligand, IL-2, GMCSF, Granzyme B, ICAM-1, and/or CXCL11. In certain aspects, the analytes are M2 factors, such as IL-4, IL-10, IL-21, VEGF, CCL5, IL-17, IL1-RII, GCSG, CXCL5.

[0026] In certain aspects, the method comprises a culture with TREM2 wild-type microglia, a culture with TREM2 heterozygous knockout microglia, and/or a culture with TREM2 homozygous knockout microglia.

[0027] In some aspects, measuring functional activity comprises measuring neural function by calcium signaling or microelectrode array (MEA). In certain aspects, measuring functional activity comprises measuring amyloid beta phagocytic function.

[0028] In particular aspects, the neurodegenerative disease is Alzheimer's disease or multiple sclerosis.

[0029] A further embodiment provides the use of the culture of the present embodiments and aspects thereof (e.g., cell culture comprising induced pluripotent stem cell (iPSC)-derived microglia, astrocytes, and/or neurons in media or cell culture comprising brain microvascular endothelial cells (BMECs), pericytes, and astrocytes in a sandwich format) as a model of a neurodegenerative disease.

[0030] In some aspects, the model comprises a culture with TREM2 wild-type microglia, a culture with TREM2 heterozygous knockout microglia, and/or a culture with TREM2 homozygous knockout microglia. In certain aspects, the model comprises engineered or patient specific iPSC derived astrocytes, neurons and/or microglia harboring disease relevant genotypes SNPs or mutations in APOE4/4, CD33, ABCA, BIN1, or R47H.

[0031] Another embodiment provides a method of screening for a neurodegenerative disease comprising detecting a level of soluble TREM2 in a culture of the present embodiments and aspects thereof (e.g., cell culture comprising induced pluripotent stem cell (iPSC)-derived microglia, astrocytes, and/or neurons in media or cell culture comprising brain microvascular endothelial cells (BMECs), pericytes, and astrocytes in a sandwich format).

[0032] In some aspects, cells in the culture are derived from isogenically engineered iPSC lines or from donors expressing disease associated SNPs or mutations associated with neurodegeneration. In particular aspects, the level of soluble TREM2 is detected in conditioned media. In some aspects, detecting comprises performing an ELISA. In some aspects, detecting an increased level of soluble TREM2 as compared to a control indicates the presence of a neurodegenerative disease. In some aspects, the method further comprises detecting the level of COMT, NRXN2 and/or SST in microglia. In specific aspects, the neurodegenerative disease is Alzheimer’s disease or multiple sclerosis.

[0033] Further embodiments of the present disclosure provides methods and compositions for an *in vitro* method for differentiating induced pluripotent stem cells (iPSCs) comprising: (a) culturing the iPSCs on a charged surface in the absence of extracellular matrix proteins; and (b) differentiating the iPSCs to endothelial cells, mesenchymal stem cells (MSCs), or hematopoietic precursor cells (HPCs).

[0034] In some aspects, the charged surface is positively charged. In certain aspects, the positively charged surface is an amine surface or Poly L Lysine surface. In specific aspects, the positively charged surface comprise nitrogen-containing functional groups. In other aspects, the charged surface is negatively charged. In particular aspects, the negatively charged surface is a carboxyl surface. In certain aspects, the negatively charged surface comprise oxygen-containing functional groups. In some aspects, the charged surface is a polymeric surface. For example, the polymeric surface is a polystyrene surface. In certain aspects, the charged surface comprises positively charged groups and negatively charged groups. In some aspects, the positively charged groups are nitrogen-containing groups and the negatively charged groups are oxygen-containing groups.

[0035] In certain aspects, the iPSCs are cultured in serum free defined media. In some aspects, differentiating comprises culturing in the presence of blebbistatin or a ROCK inhibitor, such as H1152. In particular aspects, the method is free of or essentially free of extracellular matrix proteins, such as laminin, fibronectin, vitronectin, MATRIGEL™, tenascin, entactin, thrombospondin, elastin, gelatin, or collagen.

[0036] In additional aspects, the method further comprises engineering the iPSCs to have disrupted expression of TREM2, MeCP2, and/or SCNA prior to step (a). In particular aspects, engineering comprises using a TAL nuclease to introduce indels in exon 2 of TREM2. In some aspects, the disrupted expression of MeCP2 is further defined as a truncated mutant of MeCP2 protein. In certain aspects, the disrupted expression is due to a missense point mutation, such as A53T.

[0037] In some aspects, the method comprises differentiating the progenitor cells to endothelial cells. In certain aspects, step (a) comprises culturing on an amine surface to generate progenitor cells and step (b) comprises culturing on a carboxyl surface in the presence of endothelial differentiation media to produce endothelial cells. In particular aspects, the endothelial cells are positive for CD31.

[0038] In further aspects, the method further comprises differentiating the endothelial cells to brain microvascular endothelial cells (BMECs).

[0039] In some aspects, the method further comprises differentiating the endothelial cells to lymphatic endothelial cells.

[0040] In certain aspects, the method comprises differentiating the progenitor cells to MSCs. In specific aspects, differentiating comprises culturing the progenitor cells on an amine surface in the presence of MSC media. In some aspects, the MSCs are positive for CD73, CD44, and CD105. In certain aspect, at least 90% of the differentiated cells are positive for CD73.

[0041] In additional aspects, the method further comprises differentiating the MSCs to pericytes. In certain aspects, the MSCs are cultured in the presence of pericyte medium in the absence of extracellular proteins. In some aspects, the pericytes are positive for NG2, PDGFRβ, and CD146.

[0042] In some aspects, the method comprises differentiating the progenitor cells to HPCs. In certain aspects, the method further comprises differentiating the HPCs to microglia. In specific aspects, differentiating comprises culturing the HPCs on a neutrally charged surface or ultralow attachment surface in the presence of microglia differentiation media. In certain aspects, the microglia differentiation media comprises IL34, TGF, and MCSF or analogs or mimetics thereof. In some aspects, differentiating comprises culture at normoxia. In some aspects, differentiating is for 20-25 days. In particular aspects, the microglia are positive for CD45, CD11b, and CD33. In certain aspects, at least 50% (e.g., 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 50-60%, 60-70%, or 80-90%) of the differentiated cells are positive for CD11b. In some aspects, at least 90% (e.g., 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) of the differentiated cells are positive for CD33.

[0043] In certain aspects, the method does not comprise purification of the cells. In some aspects, purification is further defined as performing MACS.

[0044] In particular aspects, the method is good-manufacturing practice (GMP) compliant. In some aspects, the method is performed under hypoxic conditions. In specific aspects, the iPSCs are human.

[0045] In another embodiment, there is provided a composition comprising a microglia cell population at least 90% (e.g., 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100%, 90-93%, 93-96%, or 96-100%) positive for P2RY12, CX3CR1, TMEM119, IBA-1 and TREM2. In some aspects, the microglia cell population is produced by the method of the embodiments. In some aspects, the microglia cell population is generated from disease associated iPSC donors with TREM2, APOE, CD33, BIN, ABCA7, SNPS or genotypes associated with neurodegeneration. In certain aspects, the microglia cell population has disrupted expression of TREM2, MeCP2, and/or SCNA. In some aspects, the disrupted expression of TREM2 is further defined as a homozygous knockout of TREM2 expression. In certain aspects, the disrupted expression of MeCP2 is further defined as a truncated mutant of MeCP2 protein. In some aspects, the disrupted expression of SCNA is due to a missense point mutation, such as A53T.

[0046] A further embodiment provides a method for screening a test compound comprising introducing the test compound to a microglia cell population of the present embodiments. In some aspects, the microglia cell population is further introduced to amyloid-beta. In certain aspects, the microglia cell population is further introduced to LPS.

[0047] Another embodiment provides a composition comprising a pericyte cell population produced by the method of the present embodiments.

[0048] In yet another embodiment, there is provided herein a blood-brain-barrier model comprising microglia, pericytes, and BMECs produced by the present embodiments.

[0049] A further embodiment provides a method for generating microglia comprising: (a) differentiating iPSCs to HPCs; and (b) sorting the HPCs for CD34-positive cells; and (c) culturing the HPCs in Microglia Differentiation Medium, thereby generating a population of microglia. In some aspects, the HPCs are differentiated according to the present embodiments. In certain aspects, sorting comprises using CD34 magnetic beads. In particular aspects, the method does not comprise sorting HPCs for CD43-positive cells. In particular aspects, the method does not comprise ECM proteins.

[0050] In some aspects, the Microglia Differentiation Medium comprises IL-34, TGFβ1, or M-CSF or the respective analogs or mimetics thereof. In certain aspects, the Microglia Differentiation Medium comprises 200 ng/mL IL-34, 100 ng/mL TGFβ1, and 50 ng/mL M-CSF. In some aspects, the cells are fed with Microglia Differentiation Medium every 48 hours.

[0051] In particular aspects, at least 90% (e.g., 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100%, 90-93%, 93-96%, or 96-100%) of the cells in the population of microglia are TREM-positive. In some aspects, at least 10% (e.g., 15%, 20%, 25%, 30%, 10-15%, 15-20%, or 20-30%) of the HPCs are differentiated to microglia.

[0052] In certain aspects, the culturing of step (b) is performed in a 96 well format. In certain aspects, the culturing of step (b) is performed on a charged surface. In some aspects, the charged surface is positively charged. For example, the positively charged surface is an amine surface. In other aspects, the charged surface is negatively charged. For example, the negatively charged surface is a carboxyl surface.

[0053] In additional aspects, the method further comprises maturing the population of microglia in media comprising CD200 and/or fractalkine or analogs or mimetics thereof. In some aspects, the method further comprises cryopreserving the microglia.

[0054] In some aspects, the HPCs are differentiated from iPSCs engineered to have disrupted expression of TREM2. In certain aspects, engineering comprises using a TAL nuclease.

[0055] In certain aspects, the cryopreserved microglia retain phagocytic function towards pHrodo bacterial particles. In particular aspects, the cryopreserved microglia can mature post thaw and respond to stimulants and secrete interleukins, chemokines and immune modulating ligands in the supernatant media.

[0056] A further embodiment provides an *in vitro* method for producing neural progenitor cells (NPCs) from iPSCs comprising (a) pre-conditioning iPSCs in media comprising a glycogen synthase kinase 3 (GSK3) inhibitor; (b) differentiating the iPSCs to NPCs, wherein the method does not comprise inhibition of SMAD signaling.

[0057] In some aspects, the iPSCs are maintained under hypoxic conditions prior to step (a). In certain aspects, the iPSCs are seeded in the presence of a ROCK inhibitor and then cultured in the absence of a ROCK inhibitor prior to step (a).

[0058] In certain aspects, the GSK3 inhibitor is CHIR99021, BIO, or SB-216763. In particular aspects, the GSK3 inhibitor is CHIR99021, such as at a concentration of 1 μM, 2 μM, 3 μM, 4 μM, or 5 μM, particularly 3 μM. In some aspects, the pre-conditioning is for 2-4 days, such as 1, 2, or 3 days.

[0059] In some aspects, the iPSCs of step (a) and/or step (b) are cultured on an extracellular matrix (ECM) protein-coated surface. In certain aspects, the ECM protein is

MATRIGEL™, laminin, or vitronectin. In specific aspects, the ECM protein is laminin or vitronectin.

[0060] In certain aspects, steps (a) and (b) are performed under normoxic conditions. In some aspects, differentiating comprises culturing the iPSCs on an ECM protein-coated surface. In certain aspects, the ECM protein is laminin or vitronectin. In some aspects, differentiating comprises culturing the iPSCs on an ultralow attachment plate or spinner flask in the presence of a ROCK inhibitor. In particular aspects, step (b) is performed for 5 to 10 days, such as 6 days, 7 days, 8 days, 9 days, or 10 days.

[0061] In additional aspects, the method further comprises detecting expression of Tra-162, CD56, CD15, Sox1, Nestin, β3 Microglobulin, and/or Pax-6 in the NPCs. In some aspects, at least 70% (e.g., 80%, 85%, 90%, 95%, 70-80%, 80-90%, or 90-100%) of the NPCs are positive for CD56.

[0062] In further aspects, the method comprises further differentiating the NPCs to astrocytes or neurons.

[0063] Another embodiment provides a method of screening for a neurodegenerative disease comprising detecting a level of soluble TREM2 in microglia conditioned media. In some aspects, detecting comprises performing an ELISA. In certain aspects, the microglia are derived from isogenically engineered iPSCs or a donor expressing disease-associated SNPs or mutations. In some aspects, the microglia are produced by the methods of the present embodiments or aspects thereof. In certain aspects, an increased level of soluble TEM as compared to a control detects a neurogenerative disease, such as Alzheimer's disease or multiple sclerosis.

[0064] A further embodiment provides a method for performing high-throughput screening to identify a therapeutic agent comprising contacting microglia produced by the method of the present embodiments or aspects thereof with a plurality of candidate agents and measuring cytokine and/or chemokine levels and/or amyloid beta phagocytic function.

[0065] In some aspects, the microglia are cryopreserved microglia derived from isogenically engineered iPSC lines, microglia derived from a donor expressing disease associated SNPs, or microglia derived from a donors expressing mutation associated with neurodegeneration. In some aspects, the cytokines and/or chemokines are selected from the group consisting of IL6, IL10, IL3, TNFα, IL13, CCL2/MCP-1, CCL20/MIP-3α, CCL4/MIP-1β, CCL5/RANTES, CX3CL1/Fractalkine, CXCL1/GROα, CXCL10/IP-10, CXCL2/GROβ, and IL-8/CXCL8.

[0066] Also provided herein is a co-culture comprising microglia of the present embodiments and aspects thereof and endothelial cells, pericytes, astrocytes, and/or neural precursor cells. Another embodiment provides the use of the co-culture to mimic human brain development.

[0067] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0068] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0069] FIG. 1: Schematic representation of the 2D and 3D HPC differentiation process.

[0070] FIG. 2: Schematic representation of the derivation of endothelial cells from day 6 HPCs derived from 2D or 3D HPC differentiation process.

[0001] FIG. 3: Characterization of endothelial cells generated using successive passage purification without the use of CD31$^+$ MACS.

[0002] FIG. 4A-B: (FIG. 4A) Morphology of cells at the end of replate 3. The endothelial cells can be cryopreserved at the end of replate passage 2 or 3. **(FIG. 4B)** Media formulations used for derivation of endothelial cells.

[0003] FIGS. 5A-5D: (FIG. 5A) Overview of MSC differentiation process. **(FIG. 5B)** Media formulations for generating MSCs. **(FIG. 5C)** Schematic depicting tri lineage differentiation of MSCs to Adipocytes, Osteocytes and Cartilage. **(FIG. 5D)** Phenotypic characterization of day 6 MSC progenitor.

[0004] FIG. 6: Emergence of a pure population of MSCs on Amine surface. Cryopreserved day 6 HPCs or live cultures at the end of day 6 differentiation are placed in the presence of MSC media in the presence of 1 uM H1152 (or blebbistatin) on an Amine charged surface plates. The cultures were transitioned to normoxia and normal tissue culture plates at P4. The purity spec for MSCs was reached at P5.

[0005] FIG. 7: Cells stained for surface MSC markers CD73, CD44, CD105, CD49d, and absence of Endothelial markers CD31 and CD144. Cryopreserved day 6 HPCs or live cultures at the end of day 6 differentiation are placed in the presence of MSC media in the presence of 1 uM H1152 (or blebbistatin) on an Amine charged surface plates. The cultures were transitioned to normoxia and normal tissue culture plates at P5. The purity spec for MSCs was reached at P6.

[0006] FIGS. 8A-8C: (FIG. 8A) Tri-lineage potential. Various steps for generating adipocytes, osteocytes, and chondrocytes from MSCs. **(FIG. 8B)** Pictures demonstrating tri-lineage potential of MSCs, alizarin red staining for osteocytes, alcian blue for chondrocytes, and oil red O for adipocytes. **(FIG. 8C)** MSC plated at 1,000 cells/cm^2 in MSC Differentiation Medium and feed every other day for 10-14 days. Plates stained using crystal violet and total number of colonies counted.

[0007] FIGS 9A-9H: Conversion of MSCs to pericytes. (FIG. 9A) Schematic overview of the process of converting iCell MSCs to iPSC-derived pericytes. **(FIG. 9B)** Media formulations for generating iPSC-derived pericytes. **(FIG. 9C)** Comparative flow cytometry for known pericyte markers PDGFRβ, NG2, and CD146 in iCell MSCs, iPSC-derived pericytes, and ScienCell primary human brain vascular pericytes (HBVPs). There was an absence of pericyte markers for iCell MSCs at thaw and gain of pericyte markers by the end of P1 in pericyte medium. iPSC-derived pericytes show greater purity of pericyte specific markers than primary HBVPs **(FIG. 9D)** Morphology of iPSC-derived MSC (P2), iPSC-derived pericyte (P1), and ScienCell primary HBVP morphology via bright field microscopy. **(FIG. 9E)** Table describing differences between PC1 and PC2 pericyte subtypes based on phenotype and marker expression. **(FIG. 9F)** iPSC-derived pericytes were stained via flow cytometry immediately post-thaw and five days post thaw for pericyte subtype specific markers CD274, VCAM1, Desmin, DLK1, and αSMA, as well as generic pericyte markers PDGFRβ, NG2, CD13, and CD146. iPSC-derived pericytes reveal the signature of contractile pericytes, subtype PC2. **(FIG. 9G)** IncuCyte live imaging system images of iPSC-derived pericytes in phagocytosis assay. iPSC-derived pericytes show observable phagocytic activity of S. aureus bioparticles above control levels (A) iPSC-derived pericyte alone control. (B) iPSC-derived pericyte + NucGreen Dead 488 (NG) reagent control. (C) iPSC-derived pericyte + S. aureus pHrodo Red BioParticles (BP). (D) iPSC-derived pericyte + NucGreen Dead 488 reagent (NG) + S. aureus pHrodo Red BioParticles (BP). All images taken from time point 36 days 16 hours post cell seeding. **(FIG. 9H)** Quantification of phagocytic activity via total red object integrated intensity analyzed by IncuCyte software.

[0008] FIG. 10A-10G: Generation of brain microvascular endothelial cells (BMECs). (FIG. 10A) Schematic overview of generating brain microvascular endothelial cells. **(FIG. 10B)** Composition of ECRA medium. **(FIG. 10C)** Flow cytometric analysis of BMECs by co expression of Glut1/CD31. **(FIG. 10D)** Immunohistochemical Staining of BMECs with P Glycoprotein (Green) expression on day 13. Nuclei was stained with Hoechst3342 and the images was captured at 200X magnification by ImageXpress (Molecular Devices, LLC). **(FIG. 10E)** Functional characterization of BMECs by measuring TEER values across days post plating. **(FIG. 10F)** Schematic overview of generating brain microvascular endothelial cells using an alternate method including a preconditioning step and plating on charged surfaces without using ECM. Description of the modified media to induce the generation of BMECs on charged surfaces. **(FIG. 10G)** Day 7 differentiating brain microvascular endothelial cells on different charged surfaces were harvested and the purity was quantified by staining for the presence of CD31, p glycoprotein/ and Glut-1 expression and the absence of pluripotency marker (TRA-181) expression.

[0009] FIG. 11: Scale up of HPCs using the 3D differentiation process and subsequent purification using CD34$^+$ Magnetic beads. A schematic description of scale up and sorting of HPCs using CD34 magnetic beads. The efficiency of the sorting process via manual and CliniMACs mediated separation is illustrated. The actual purity of the HPCs (measured by the percentage of CD34 positive cells in the sorted fraction) per run and the efficiency of the process is outlined.

[0010] FIG. 12: Media formulations for microglia differentiations.

[0011] FIGS. 13A-13B: (FIG. 13A) A schematic description of derivation of microglia from CD34+ sorted HPC. HPCs were placed in microglia differentiation media MDM. The cultures were fed every 48 hours with MDM or 2X MDM. The cultures were split on day 12 of differentiation and the 2D differentiation was continued until day 23. The cells were harvested on day 23 and stained for the presence of purity markers for microglial cultures. The rest of the culture was cryopreserved. The purity of the microglia cultures was quantified before and after cryopreservation. **(FIG. 13B)** Microglia differentiation medium and microglia differentiation medium are depicted.

[0012] FIG. 14: Microglia purity assessment in the presence of MDM media before and after cryopreservation. Microglia cultures on day 23 differentiation were harvested and stained for the presence of microglia specific markers. The remaining cells were cryopreserved using a control rate freezer. The cryopreserved cells were thawed and stained for the presence of microglia specific markers. For both sets cell surface expression of CD45, CD33, TREM2, and CD11b (FIGS. 14A) as well as intracellular expression of CX3CR1 PU.1, IBA, P2RY12, TREM2 and TMEM119 by flow cytometry.

[0013] FIGS. 15A-C: Recovery of microglia post cryopreservation using manual vs control rate freezer. HPCs were placed in media to initiate microglia differentiation in the presence of MDM. The cells were cryopreserved at day 20 **(FIG. 15B)**, day 23 **(FIG. 15B),** and day 26 **(FIG. 15C)** of differentiation using manual freezing protocol or a control rate freezer (CRF). The cryopreserved cells were transferred to liquid nitrogen for a week. Cryopreserved Microglia were thawed and placed in microglia maturation medium (MMM). The cultures were fed every 48 hrs with fresh maturation media. The cells were harvested on day 3, 5, 7, 10, 12 and 14-days post thaw and the recovery of viable cells with respect to the initial plating number was quantified.

[0014] FIG. 16: Efficiency of converting HPCs to microglia. Cryopreserved HPCs were differentiated to microglia in the presence of MDM (N=4). The total viable number of input HPCs and output microglia was quantified. The process efficiency was calculated based on the purity and absolute number of TREM2 positive cells present on day 23 of microglia differentiation divided by the absolute number of input viable HPCs.

[0015] FIGS. 17A-17C: Purity analysis of microglia cryopreserved either manually or in the presence of a control rate freezer on day 20 (FIG. 17A), day 23 (FIG.

17B) and day 26 (FIG. 17C) at thaw, 3 days post thaw and 10 days post thaw. Microglia cryopreserved on day 20, 23 and 26 were thawed and plated for 3, 5, 7, 10 and 12 days in Microglia Maturation Medium. The cells were stained for the presence of Pu1, IBA, CX3CR and P2RY12 expression by flow cytometry.

[0016] FIGS. 18A-18B: Viable manual hemocytometer cell counts of microglia cryopreserved either manually or in the presence of a control rate freezer on day 0 (**FIG. 18A**), and day 3 (**FIG. 18B**), post thaw to set up the phagocytosis assays with S. aureus bioparticles.

[0017] FIG. 19: Functional Characterization of microglia cryopreserved on day 20, day 23 and day 26 of differentiation using the manual or control rate freezer. Cryopreserved Microglia were thawed and plated at 15,000 viable cells/well in a 96 well plate in the presence of 200 µl Microglia Maturation medium per well. The cells were treated with diluted 1 µg/well of opsonized or non-opsonized pHrodo Red BioParticles (Thermo Fisher # A10010, 2 mg per vial; stored at -20°C). The plate was placed on the IncuCyte and images of the phagocytosis were taken at various time points up to 5 days post thaw. Cells cryopreserved via control rate freezer method exhibit more robust phagocytosis (due to higher cell viability). Manual cryopreservation method revealed decreased / right-shifted rate of phagocytosis across all conditions (due to lower cell viability).

[0018] FIG. 20: **Functional Characterization of live day 14 microglia and microglia cryopreserved on day 20, day 23 and day 26 of differentiation using the manual or control rate freezer assessed via live imaging on the IncuCyte system.** Cryopreserved microglia were thawed and plated in MMM for three days. The viable cell counts at the end of three days were determined as described in Figure 18B. 15,000 viable cells were plated in a 96 well plate in the presence of 200 µl Microglia Maturation medium (MMM) per well. The cells were fed fresh 50µl media of MMM every 48 hours. The cells were treated with diluted 1 µg/well of opsonized or non-opsonized pHrodo Red BioParticles (Thermo Fisher # A10010, 2 mg per vial; stored at -20°C). The plate was placed on the IncuCyte and images of the phagocytosis were taken at various time points up to 5, 7- and 14-days post thaw. Manual cryopreservation method revealed decreased / right-shifted rate of phagocytosis across all conditions (due to lower cell viability).

[0019] FIG. 21: The phagocytic efficiency ratio was determined by dividing the number of phagocytic red cell count/total cell number from the post thaw sample.

[0020] FIG. 22: **Functional characterization of cryopreserved microglia using pHrodo Amyloid beta.** Cryopreserved day 23 microglia were thawed and plated at 15,000 viable cells/well in a 96 well plate in the presence of 200 µl Microglia Maturation medium per well. The cells were treated with pHrodo Amyloid beta. The control set contained cells with media without any pHrodo Amyloid beta. The plate was placed on the IncuCyte and images of the phagocytosis were taken at various time points up to 24 hours post thaw.

[0021] FIGS. 23A-23B: **Miniaturizing the differentiation of HPCs to microglia in the absence of ECM and in a 96 well format amenable to screening applications.** Schematic representation of the differentiation of HPCs to microglia (**FIG. 23A**) and the different charged surfaces used in the experiments. Ultra-low attachment (ULA), Tissue Culture (TC) and Non-tissue culture (Non-TC) vessels (**FIG. 23B**).

[0022] FIGS. 24A-24B: **End stage purity analysis of day 23 microglia in the presence of various charged surfaces.** Cryopreserved HPCs were plated at a density of 20,000-35,000 viable cells/cm^2 on a 96 well Primaria plate or Ultra-low attachment, tissue culture (TC) or non-tissue culture plates (Non-TC) in the presence of 200µl microglia differentiation medium per well. The cells were fed every 48 hrs with 50 µl media per well of MDM for the next 23 days of differentiation. The cells were harvested with cold PBS on day 23 and the total viable cell number was quantified using an automated cell counter. The cells were stained for surface expression of CD11b, CD45, CD33, TREM2 and intracellular expression of TREM2, IBA, P2RY12 and TMEM119.

[0023] FIGS. 25A-25B: **Cytokines and Chemokines released by cryopreserved microglia.** Day 23 cryopreserved microglia were thawed into MDM medium and plated on to Primaria 96 well plate at 50,000 cells/well. Cells were plated for three days prior to starting stimulations with 100 ng/ml LPS and 50 ng/ml interferon gamma. Stimulations were performed in triplicate for 24 hours. Supernatant was spun down to remove cells and debris and immediately placed at -20°C. The supernatants were analyzed on a multiplex Luminex assay. Average of multiple WT batches **(FIG. 25A)**, and WT, Homozygous and Heterozygous TREM2 Knockouts (KOs), MECP2 HM and A53T-SNCA engineered lines **(FIG. 25B)** are shown.

[0024] FIG. 26: Cryopreserved microglia derived from multiple batches of Homozygous and Heterozygous TREM2 Knockouts (KOs), MECP2 and A53T-SNCA engineered lines were stained for the presence of surface expression of CD11b, CD45, TREM2, as well as intracellular markers PU.1, IBA-1, CX3CR1, P2RY12 and TMEM119. The engineered iPSC lines revealed comparable expression of TREM2 by flow cytometry.

[0025] FIGS. 27A-27B: (FIG. 27A) Release of soluble TREM2 in cryopreserved Microglia derived from Wildtype (WT), Heterozygous (HT) and Homozygous (HO) TREM2 KO engineered iPSCs. Levels of soluble TREM2 (sTREM2) were quantified from conditioned media from WT and TREM2 Heterozygous and Homozygous KO mutants using a Simple Step ELISA (AbCam). WT and TREM2 KO microglia were thawed and plated at the same density in maturation media in a 96 well Primaria plate. The spent media was collected at Day 3 post thaw and Day 7 post thaw. The cultures were half fed with fresh maturation media on Day 3 and Day 5 post thaw. **(FIG. 27B) Release of soluble TREM2 in cryopreserved microglia derived from Wildtype (WT), MECP2 HM and A53T-SNCA engineered iPSCs.** Levels of soluble TREM2 (sTREM2) were quantified from conditioned media from WT, MECP2 HM and A53T-SNCA engineered lines using a Simple Step ELISA (AbCam). Microglia were thawed and plated at the same density in maturation media in a 96 well Primaria plate. The spent media was collected at Day 3 post thaw and Day 7 post thaw. The cultures were half fed with fresh maturation media on Day 3 and Day 5 post thaw.

[0026] FIG. 28: **List of media formulations to study the survival kinetics of WT, HT and HO TREM2 KO engineered microglia WT, 1185 HT TREM2 KO, 1187 HO TREM2 KO.** Microglia were placed at a density of 15,000 viable cells per well in a 96 well plate in 250 µl of the microglia base medium containing 32 different variations of cytokine formulations. The kinetics of cell survival was captured on the IncuCyte system. NucGreen Dead diluted to 2 drops/mL was added to all wells containing cells with various media compositions to capture the number of dead cells with time. The images were captured every 8 hours and the experiment continued for 72 hours without any intermittent feeds.

[0027] FIGS. 29A-29C: WT **(FIG. 29A),** 1185 HT TREM2 KO **(FIG. 29B),** 1187 HO TREM2 KO **(FIG. 29C)** Microglia cell survival kinetics.

[0028] FIGS. 30A-30C: WT **(FIG. 30A),** 1185 HT TREM2 KO **(FIG. 30B),** 1187 HO TREM2 KO **(FIG. 30C)** Microglia cell survival kinetics with two cytokines.

[0029] FIGS. 31A-31C: WT **(FIG. 31A),** 1185 HT TREM2 KO **(FIG. 31B),** 1187 HO TREM2 KO **(FIG. 31C)** Microglia cell survival kinetics with three cytokines.

[0030] FIGS. 32A-32C: WT **(FIG. 32A),** 1185 HT TREM2 KO **(FIG. 32B),** 1187 HO TREM2 KO **(FIG. 32C)** Microglia cell survival kinetics with four cytokines.

[0031] FIGS. 33A-33E: WT, 1185 HT TREM2 KO, 1187 HO TREM2 KO Microglia were placed at a density of 15,000 viable cells in a 96 well plate in 250 µl of Microglia base medium **(FIG. 33A)** or MMM **(FIG. 33B),** or Microglia base medium supplemented with IL-34 **(FIG. 32C),** or Microglia base medium supplemented with IL-34 **(FIG. 33D),** or Microglia base medium supplemented with MCSF **(FIG. 33D)** or base medium supplemented with only IL-34 **(FIG. 33C)** or MSCF or a combination of IL-34 and MCSF **(FIG. 33E).** The kinetics of cell survival was captured on the IncuCyte system. NucGreen Dead diluted to 2 drops/mL was added to all wells containing cells with various media compositions to capture the number of dead cells with time. The images were captured every 8 hours and the experiment continued for 7 days without any intermittent feeds. The intensity of the NucGreen Dead quantifies the dead cells in the cultures.

[0032] FIGS. 34A-34H: Functional Characterization of WT, 1185 HT TREM2 KO, 1187 HO TREM2 KO microglia, cryopreserved on day 23 with pHrodo Red labelled bacterial BioParticles and pHrodo Red Amyloid beta. WT, 1185 HT TREM2KO, 1187 HO TREM2 KO microglia were plated at a density of 15,000 viable cells/cm^2 in a 96 well plate in 250 µl of MMM (**FIGS. 34A-B**) or MDM base (AKA microglia base medium) supplemented only MSCF (**FIGS. 34C-D**) or IL-34 (**FIGS. 34E-F**) or a combination of IL-34 and MCSF (**FIGS. 34G-H**) for three days post thaw. The cells were treated with diluted 1 µg/well of opsonized or non-opsonized pHrodo Bioparticles (Thermo Fisher # A10010, 2 mg per vial; stored at -20C) (**FIGS. 34 A, C, E, G**) or pHrodo Amyloid beta (**FIGS. 34B, D, F, H**). The plate was placed on the IncuCyte and image of the phagocytosis were taken at various time points up to 30 hrs. WT as well as engineered microglia revealed phagocytic function post thaw. The kinetics and the efficiency of phagocytosis varied between the WT, 1185 HT TREM2 KO, 1187 HO TREM2 KO microglia.

[0033] FIG. 35: **Purity of microglia cultures in a Simplified Maturation Medium.** Post thaw purity of day 23 cryopreserved Wild Type (WT) microglia in the presence of MMM or microglia base medium supplemented with combinations of two critical (IL-34, MSCF) cytokines in the maturation media. The purity was quantified at day 3, 7 and day 14 post thaw by harvesting the cells and the purity of CD45, CD33, TREM2, CD11b, CX3CR1, P2RY12, TMEM119, IBA, was determined at the end of the differentiation process by harvesting the cells and staining the cells for cell surface and intra cellular staining of markers by flow cytometry. Cryopreserved microglia retain viability, purity in maturation media supplemented with MSCF and IL-34. This simplified media will be valuable for co culture applications of cryopreserved microglia with neurons and astrocytes for developing TREM a brain organoids.

[0034] FIGS. 36A-36B: (**FIG. 36A**) Schematic depicting screening experiment with cryopreserved microglia. (**FIG. 36B**) Table of compounds tested in screen.

[0035] FIGS. 37A-37D: Results of microglia screening experiment with GW501516 (**FIG. 37A**), Leucettine L41 (**FIG. 37B**), Piceatannol (**FIG. 37C**), and Azeliragon (**FIG. 37D**).

[0036] FIGS. 38A-38D: Results of microglia screening experiment with J147 (**FIG. 38A**), Dibutryl-cAMP (**FIG. 38B**), Isradipine (**FIG. 38C**), and Bexarotene (**FIG. 38D**).

[0037] FIGS. 39A-39E: Results of microglia screening experiment with SB-431542 (**FIG. 39A**), SP600125 (**FIG. 39B**), GW2580 (**FIG. 39C**), PP2 (**FIG. 39D**), and SB239063 (**FIG. 39E**)

[0038] FIGS. 40A-40D: Results of microglia screening experiment with GW501516 (**FIG. 40A**), Leucettine L41 (**FIG. 40B**), Piceatannol (**FIG. 40C**), and Azeliragon (**FIG. 40D**) with LPS stimulation.

[0039] FIGS. 41A-41E: Results of microglia screening experiment with J147 (**FIG. 41A**), Dibutryl-cAMP (**FIG. 41B**), Isradipine (**FIG. 41C**), Bexarotene (**FIG. 41D**), and SB-43152 (**FIG. 41E**) with LPS stimulation.

[0040] FIGS. 42A-42D: Results of microglia screening experiment with SP600125 (**FIG. 41A**), GW2580 (**FIG. 42B**), PP2 (**FIG. 42C**), and SB239063 (**FIG. 42D**) with LPS stimulation.

[0041] FIG. 43: Summary of microglia screening experiment results.

[0042] FIGS. 44A-44G: (**FIG. 44A**) Microglia with ATP/BzATP All Traces – Ratio. (**FIG. 44B**) Microglia with ATP/BzATP Sample Traces – Ratio. (**FIG. 44C**) Response to BzATP in microglia. (**FIG. 44D**) Differential Response to ADP in microglia. (**FIG. 44E**) Response with 100μM BzATP in the presence of P_2X_7 antagonist AZ11645373. (**FIG. 44F**) Response with 100μM BzATP in the presence of P_2X_7 antagonist A438079. (**FIG. 44G**) Dose dependent response to demonstrate functional ADP dependent response in microglia in the presence of AZD1283 (a potent antagonist of the P2Y$_{12}$ receptor).

[0043] FIGS. 45A-45B: Release of soluble TREM2 in cryopreserved microglia derived from ANH and Disease Associated Microglia were quantified from conditioned media collected on day 3 (**FIG. 45A**) or day 7 (**FIG. 45B**) post thaw using a Simple Step ELISA (AbCam). ANH and DAM associated microglia were thawed and plated at the same density in maturation media in a 96 well Primaria plate. The spent media was collected at Day 3 post thaw and Day 7 post thaw. The cultures were half fed with fresh maturation media on Day 3 and Day 5 post thaw.

[0044] FIGS. 46A-46J: Cytokines and Chemokines released by cryopreserved microglia derived from ANH and Disease Associated Microglia from a panel of iPSC donors. Day 23 Cryopreserved microglia were thawed into MDM medium and plated on to Primaria 96 well plate at 50,000 cells/well. Cells were plated for three days prior to starting stimulations with 100 ng/ml LPS or IL-4 + dBu-cAMP. Stimulations were performed in triplicate for 24 hours. Supernatant was spun down to remove cells and debris and immediately placed at -20°C. The supernatants were analyzed on a multiplex Luminex assay. Upon stimulation with LPS, release of M1 analytes **(FIG. 46A)**, M2 analytes **(FIG. 46B)**, interleukins **(FIG. 46C)**, chemokines **(FIG. 46D)**, and other analytes **(FIG. 46E)**. Upon stimulation with IL-4 + dBu-cAMP, release of M1 analytes **(FIG. 46F)**, M2 analytes **(FIG. 46G)**, interleukins **(FIG. 46H)**, chemokines **(FIG. 46I)**, and other analytes **(FIG. 46J)**.

[0045] FIGS. 47A-47J: Demonstration of phagocytic function of AHN and Disease associated microglia using pHrodo labeled Staphylococcus aureus Bioparticles and Amyloid Beta: Cryopreserved AHN and Disease associated microglia were plated at 5,000 cells per well of 384 well Poly-D-Lysine plate. S. aureus bioparticles were added to each well at 0.5 μg/mL, Amyloid Beta was added at 1 μM/well. Kinetics of phagocytosis of S. aureus bioparticles and Amyloid Beta was quantified using Total Red Object Integrated Intensity using the IncuCyte Live-Cell Analysis System. (**FIG. 47A**) (ANH, S. aureus), (**FIG. 47B**) (ANH, Amyloid Beta), (**FIG. 47C**) (R47H Vs ANH, S. aureus), (**FIG. 47D**) (R47H Vs ANH, Amyloid Beta), (**FIG. 47E**) (CD33 Vs ANH, S. aureus), (**FIG. 47F**) (CD33 Vs ANH, Amyloid Beta), (**FIG. 47G**) (ABCA7 vs ANH, S. aureus), (**FIG. 47H**) (ABCA7 vs ANH, Amyloid Beta), (**FIG. 47I**) (APOE isoforms Vs ANH, S. aureus), (**FIG. 47J**) (APOE isoforms Vs ANH, Amyloid Beta).

[0046] FIGS: 48A-48D: **(FIG. 48A)** A schematic description of the method to generate Neural Precursor Cells (NPCs) from iPSCs without using dual SMAD inhibition. The various steps involved, and the composition of medias used is described. **(FIG. 48B)** Summary of the kinetics of emergence of NPCs across three iPSC lines. The decrease in pluripotency markers and the emergence of NPCs specific markers on different days of the differentiation process. Quantification of purity performed by cell surface straining and intracellular staining by flowcytometry **(FIG. 48C)** Staining for Astrocytes derived from NPC across multiple passages in cultures. Purity of astrocytes quantified by cell surface and intracellular staining by flow cytometry. **(FIG. 48D)** Differentiation of NPCs to neurons and quantification of purity of end stage neurons by intracellular flow cytometry.

[0047] FIG. 49: Summary of surfaces compatible for derivation of iPSC derived cell lineages.

[0001] FIGS. 50A-50D: Immunohistochemical staining of 14 day (**FIG. 50A**) mono-culture, (**FIG. 50B**) bi-culture, and (**FIG. 50C**) tri-culture generated from cryopreserved microglia, gabanergic neurons or glutanergic neurons and astrocytes in the presence of the triculture media. 14 day cultures were fixed using (PFA), and stained using Pan Neuronal Marker (1:1500; Millipore, Catalog: MAB2300), Anti-Iba1 (1:500; Wako Chemicals, Catalog: 019-19741), and Anti-GFAP (1:500; Abcam, Catalog: ab4674) diluted in Blocking Buffer. Following primary incubation the cells were stained with secondary antibody solutions Goat anti-Mouse IgG1 Alexa Fluor 488 (1:1000; Invitrogen, Catalog: A21121), Goat anti-Rabbit IgG Alexa Fluor 568 (1:1000; Invitrogen, Catalog: A11011), Goat anti-Chicken IgY Alexa Fluor 647 (1:1000; Invitrogen, Catalog: A21449), and Hoechst 33342 (1:10,000; Thermo Scientific, Catalog: 62249) diluted in Blocking Buffer. The plate was washed and imaging was performed on an ImageXpress Micro Confocal High-Content Imaging System (Molecular Devices). (**FIG. 50D**) Comparison of media supplements.

[0002] FIG. 51: Tri-culture model of microglia, neurons, and astrocytes.

[0003] FIG. 52: Schematic description of setting up a triculture model to study neuroinflammation.

[0048] FIG. 53: Comparative analysis of cytokines and chemokines released in mono-, bi- and tri-cultures with gabanergic neurons, astrocytes and microglia derived from ANH, TREMHZ and TREM2HO. The analytes released by reactive and non-reactive astrocytes were quantified in mono-, bi- and tri-cultures.

[0004] FIG. 54: Comparative analysis of M1 cytokines and chemokines released in mono-, bi- and tri-cultures with GABA neurons, astrocytes and microglia derived from ANH, TREMHZ and TREM2HO.

[0005] FIG. 55: Comparative analysis of M2 cytokines and chemokines released in mono-, bi- and tri-cultures with GABA neurons, astrocytes and microglia derived from ANH, TREMHZ and TREM2HO.

[0006] FIG. 56: Comparative analysis of C3 complement released in mono-, bi- and tri-cultures with GABA neurons, astrocytes and microglia derived from ANH, TREMHZ and TREM2HO.

[0007] FIGS. 57A-57B: Representative example of the impact of microglia on neuronal electrophysiology when cultured with Gluta neurons and astrocytes and measured by MEA functional assay. (**FIG. 57A**) Gluta neurons and astrocytes on MEA. (**FIG. 57B**) Addition of microglia to model neural networks.

[0008] FIG. 58: Representative images of microglia (AHN and TREM2HZKO) phagocytosis of pHrodo Red-labeled amyloid beta fibrils mono-culture and tri-culture taken from a corresponding kinetic phagocytosis assay with TREM2HZKO microglia.

[0009] FIG. 59: Phase contrast image of a 3D triculture with cryopreserved iPSC-derived Gluta neurons, astrocytes and microglia with the cell ratios of all three cell types

[0010] FIG: 60: Comparative analysis of Calcium transients derived from 3D culture of cryopreserved Gluta neurons in the presence and absence of microglia and astrocytes.

[0011] FIG: 61: Schematic description of the various steps to set up a sandwich co-culture with iPSC-derived cryopreserved BMECs, pericytes and astrocytes.

[0012] FIG. 62 TEER function of cryopreserved BMECs. iPSC-derived BMECs were cryopreserved, thawed, and seeded onto FN/ColIV-coated Corning transwell inserts at 1.3 x106 cells/cm^2 in BMEC media. TEER measurements were collected on Days 3 through 8 (following day numbering scheme on sandwich transwell co-culture described in FIG. 61).

[0013] FIG. 63: TEER function of cryopreserved BMECs and pericytes. iPSC cryopreserved iPSC-derived pericytes were seeded on Day 0 at three different densities (666k/cm^2, 333k/cm^2, 500k/cm^2) on the basolateral membrane. iPSC-derived BMECs were cryopreserved, thawed, and seeded on Day 1 on the apical transwell membrane at 1.3x10^6 cells/cm^2 or 1.0x10^6 cells/cm^2 in BMEC media. TEER measurements were collected on Days 3 through 8 (following day numbering scheme on sandwich transwell co-culture described in FIG. 61).

[0014] FIG. 64: TEER function of cryopreserved BMECs and astrocytes. Cryopreserved astrocytes were seeded on Day 0 at three different densities (666k/cm^2, 333k/cm^2, 500k/cm^2) on the basolateral membrane. iPSC derived BMECs were cryopreserved, thawed, and seeded on Day 1 on the apical transwell membrane at 1.3x10^6 cells/cm^2 or 1.0x10^6 cells/cm^2 in BMEC media. TEER measurements were collected on Days 3 through 8 (following day numbering scheme on sandwich transwell co-culture described in FIG. 61).

[0015] FIG. 65: TEER function of cryopreserved BMECs, pericytes and astrocytes: iPSC derived pericytes and iCell astrocytes were seeded on Day 0 at three different densities (666k/cm^2, 333k/cm^2, 500k/cm^2) on the basolateral membrane. iPSC derived BMECs were thawed and seeded on Day 1 on the apical transwell membrane at 1.3 x10^6 cells/cm^2 or 1.0 x10^6 cells/cm^2 in BMEC media. TEER measurements were collected on Days 3 through 8 (following day numbering scheme on sandwich transwell co-culture described in FIG. 61).

[0049] FIG. 66: Schematic of exemplary tri-culture protocol for culture of neurons, astrocytes, and microglia.

[0016] FIG. 67: TREM2 partial loss of function leads to reduced cholesterol and fatty acid biosynthesis. RNAseq analysis of isogenic gene-edited heterozygous and homozygous TREM2KO identified a specific phenotype attributed to partial loss not captured in homozygous TREM2 loss of function studies. As shown, partial loss of TREM2 function enhanced the down-regulation of SREBF2, the master regulator of cholesterol biosynthesis resulting in a decrease in the cholesterol and fatty acid synthome that coincides with increased lipid efflux.

[0050] FIG. 68: Gas6/Axl axis is dependent on TREM2 in a dose-dependent manner.

[0051] FIG. 69: Siglec 11 expression is affected by partial loss of TREM2 function.

[0052] FIGS. 70A-70E: (**FIG. 70A**) TREM2 regulates GRN expression in microglia. (**FIG. 70B**) TREM2 regulates expression of other ion channels in microglia. (**FIG. 70C**) TREM2 regulates expression of ligand gated ion channels in microglia. (**FIG. 70D**) TREM2 downregulates expression of voltage gated ion channels in microglia. (**FIG. 70E**) TREM2 upregulates expression of voltage gated ion channels in microglia.

[0053] FIGS. 71A-71B: (**FIG. 71A**) TREM2 downregulates expression of GPCRs in microglia. (**FIG. 71B**) TREM2 upregulates expression of GPCRs in microglia.

[0054] FIGS. 72A-72B: (**FIG. 72A**) TREM2 downregulates expression of transport proteins in microglia. (**FIG. 72B**) TREM2 upregulates expression of transport proteins in microglia.

[0055] FIGS. 73A-73B: (**FIG. 73A**) TREM2 downregulates expression of catalytic receptors in microglia. (**FIG. 73B**) TREM2 upregulates expression of catalytic receptors in microglia.

[0056] FIGS. 74A-74C: (**FIGS. 74A-74B**) Effect of TREM2 on regulating the turnover of cellular enzymes and thus effecting metabolism and function. TREM2 downregulates expression of enzymes in microglia. (**FIG. 74C**) TREM2 upregulates expression of enzymes in microglia

[0057] FIG. 75: TREM2 upregulates expression of nuclear hormone receptors in microglia.

[0058] FIGS. 76A-76B: (**FIG. 76A**) TREM2 downregulates expression of other proteins expressed in microglia. (**FIG. 76B**) TREM2 upregulates expression of other proteins expressed in microglia.

[0059] FIGS. 77A-77B: Comparative RNAseq signature of microglia harboring WT TREM2 with isogenic microglia with (**FIG. 77A**) TREM2 HZ and (**FIG. 77B**) TREM2 HO reveals a significant downregulation of multiple transcripts.

[0060] FIG. 78: **Spare Respiratory Capacity of TREM2 iCell Microglia in Mono-Culture**. iCell Microglia (MGL) were thawed and matured for three days prior to seeding for Agilent Seahorse XFe96 Mito Stress Test Assay. Microglia were seeded at 30,000 cells per well on a poly(ethylenimine) and Geltrex-coated 96-well Seahorse XF Pro M Cell Culture plate in maintenance medium and cultured overnight. On the day of assay, medium was exchanged for Assay Medium containing Seahorse XF DMEM, Glucose (10 mM), Sodium Pyruvate (1 mM), and L-Glutamate (2 mM). The plate was then incubated in a 37°C incubator with ambient CO2 for one hour. Stock compounds at 10x final concentration of Oligomycin A (10 uM), FCCP (30 uM), and Rotenone/Antimycin A (5 uM) from an Agilent Cell Mito Stress Test Kit were prepared in Assay Medium. Injection ports of a XF Pro Sensor Cartridge were loaded according to Manufacturer's Instructions with Oligomycin A, FCCP, Rotenone/Antimycin A. Samples were analyzed using an Agilent Seahorse XF Pro Analyzer using a Wave Controller software package. Cell number was determined post-assay using Hoechst nuclear dye (1:1000) and captured using an ImageXpress MetaXpress High Content Imager. Data is normalized as oxygen consumption rate (OCR) per cell. Wild type microglia consistently responded to metabolic respiration interrogation compounds with a higher spare respiratory capacity when compared to the TREM2 heterozygous and homozygous cell lines.

[0061] FIGS. 79A-79C: (**FIG. 79A**) iCell microglia misplay uniform marker expression Across iPSC backgrounds. (**FIG. 79B**) TREM2 knockouts display reduced soluble TREM2. (**FIG. 79C**) TREM2 knockouts display reduced phagocytic function.

[0062] FIGS. 80A-80B: Differential Gene Expression of TREM2 R47H. (**FIG. 80A**) Pathway analysis revealed upregulation of postsynaptic membrane receptor levels and downregulation of many RNA translation pathways. (**FIG. 80B**) TREM2 R47H and TREM2 HZ Have Reduced Expression of Key Genes in Cholesterol Biosynthesis Pathways. Heterozygous mutations in TREM2 lead to increased AD risk whereas homozygous mutations lead to the neurological condition known as Nasu-Hakola Disease.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0063] Interactions between cells of the central nervous system, such as neurons, astrocytes, and microglia as well brain microvascular endothelial cells (BMECs) and pericytes influence neuroinflammatory responses to insult in the central nervous system. *In vitro* astrocyte and microglia cultures are powerful tools to study specific molecular pathways involved in neuroinflammation; however, in order to better understand the influence of cellular crosstalk on neuroinflammation, multicellular culture models are needed.

[0064] Accordingly, in certain embodiments, the present disclosure provides multicellular *in vitro* culture models for the study of neuroinflammation, such as to identify novel targets, biomarkers, and therapeutic agents for the diagnosis, prognosis, and treatment of neurodegenerative diseases. In one embodiment, there is provided a triculture of microglia, astrocytes and neurons. In another embodiment, there is provided blood brain barrier model comprising BMECs, astrocytes, and pericytes.

[0065] Further provided herein are assays for studying neuroinflammation using the present cell culture models. The outcome of the triculture system or BBB model can be survival, synaptic pruning, microglia function by Aβ aggregation, p-tau formation, MEA function of neurons, analytes released in the media to trigger the neural inflammation cascade, and cross talk between all three cell types.

[0066] GWAS in AD identified TREM2 as a key modulator of AD risk. Heterozygous mutations in TREM2 lead to increased AD risk whereas homozygous mutations lead to the neurological condition known as Nasu-Hakola Disease. Therefore, while in each case the CNS is affected, the pathobiology and the clinical manifestations of a heterozygous versus homozygous mutation are distinct. Recently, iPSC TREM2 KO microglia have been generated to study the impact of the loss of TREM2 on microglia function and its impact on AD risk (McQuade et al., 2020, Reich et al., 2021). However, it is unclear if these microglia models will properly interrogate the role of TREM2 in AD as AD does not arise from the complete loss of TREM2 but rather the partial loss of function. Therefore, to better understand the role of TREM2 mutations on AD risk, heterozygous TREM2 loss of function microglia were generated. Validation of partial loss of TREM2 microglia identified pathways unique to HetTREM2 KO supporting the notion that heterozygous TREM2 KO microglia better model the phenotype that arises from the inheritance of TREM2 mutations that lead to increased AD risk.

[0067] Accordingly, further embodiments provide an iPSC-derived "brain-in-a-dish" tri-culture model for identifying a role for TREM2 in modulating a neuroinflammatory cascade implicated in neurodegeneration as well as a role of TREM2 in microglial phagocytosis. Further, the heterozygous TREM2 knockout microglia may be used to identify AD molecular biomarkers. Heterozygous TREM2 knockout microglia can be used to identify changes in the metabolome, lipidome, and secretome, including biomolecules found in microglia-derived extracellular vesicles released by microglia that reflect the pathway changes identified in the present studies that can serve as a biomarker of AD progression as well as phenotypic rescue following drug treatment. For example, the present studies identified perturbation in cholesterol biosynthesis pathways by downregulation of TREM2, dose dependent decrease of the Gas6-Axl axis via TREM2 perturbation, partial loss of Siglec-11 via TREM2HZ which may overcome the neuroprotective effect of CD33, upregulation of ligand gated ion channels in microglia, changes in expression of voltage gated ion channels, GPCRs, catalytic receptors, enzymes, and nuclear receptors in microglia, and a direct link between down regulation of TREM2 associated with downregulation of COMT, and NRXN2 and SST expression.

[0068] Further, in certain embodiments, the present disclosure provides methods for the production of cells of multiple lineages, such as endothelial cells, mesenchymal stem cells (MSCs), and hematopoietic precursor cells (HPCs), from induced pluripotent stem cells (iPSCs). Generally, the method comprises differentiating iPSCs to the various lineage cells by using a charged surface. Specifically, the differentiation method may be in the absence of extracellular matrix (ECM) proteins and allows for the self-purification by passaging, such as for the production of MSCs and endothelial cells.

[0069] In further embodiments, methods are provided for the differentiation of endothelial cells to brain microvascular endothelial cells (BMECs) or lymphatic endothelial cells, the differentiation of MSCs to pericytes, and the differentiation of HPCs to microglia. The process allows for the efficient generation of endothelial cells and MSCs without purification, such as MACS purification, or the use of ECM proteins. The process can be adapted to good manufacturing practice (GMP)-compliance.

[0070] Endothelial cells make up a network of interconnected cells in the human body that line blood vessels, lymphatic vessels, and form capillaries. Endothelial cells regulate the flow of nutrient substances and create and respond to diverse biologically active molecules and provide for potential uses in the tools space for screening compounds and drugs for vascular toxicity, vascular permeability, and therapeutic uses, including treatment of tissue ischemia and bioengineering of grafts. There have been many protocols used to derive endothelial cells. Almost all processes require a magnetic-activated cell sorting (MACs) separation using CD31 microbeads to generate a pure culture of endothelial cells.

[0071] In certain embodiments, the present disclosure provides methods to generate a pure population of endothelial cells from iPSCs, such as episomally reprogrammed iPSCs, by a two-step process. iPSC cells may be converted to hemogenic progenitor cells (HPCs) on a positively charged amine surface followed by further propagation and subsequent purification of endothelial cells in the presence of a negatively charged carboxyl surface. The endothelial cells may be derived by hemogenic endothelial cells without any MACs purification step. The cells express CD31/ CD144/CD105 at high purity and can be propagated to retain purity and cryopreserved at earlier and late passages.

[0072] Mesenchymal stem cells (MSCs) isolated from adult human tissues are capable of proliferating *in vitro* and maintaining their multipotency, making them attractive cell sources for regenerative medicine. However, the availability and capability of self-renewal under current preparation regimes are limited. iPSCs now offer an alternative, similar cell source to MSCs. Accordingly, certain embodiments of the present disclosure provide methods for differentiating MSCs from iPSCs, such as episomally reprogrammed iPSCs, by initiating mesodermal differentiation on a positively charged amine surface using GMP compatible conditions. MSCs derived by this process express all purity markers for the MSC lineage as well as displaying self-renewal and multipotency. These cells can be scaled up for clinical applications.

[0073] In further embodiments, the present disclosure provides methods for the differentiation of MSCs into pericytes (PCs). Pericytes, also known as mural cells, ensheath blood microvessels (i.e., capillaries, arterioles, and venules) and are generally understood as having an organizational or structural role in angiogenesis. Multiple criteria including location, morphology, gene or protein expression patterns, and density around vessels are used to identify immature and mature pericytes. In general, a pericyte obtained according to a method provided herein can be identified based on expression of known pericyte molecular markers such as, without limitation, PDGFRβ, desmin (DES), CD13 (ANPEP; alanyl (membrane) aminopeptidase), α-SMA, RGS5 (Regulator of G-protein Signaling 5), NG2 (also known as CSPG4; chondroitin sulfate proteoglycan 4), CD248 (endosialin), ANG-1, CD146, CD44, CD90, and CD13.

[0074] Microglia are innate immune cells of the central nervous system that perform critical roles in brain development, homeostasis, and immune regulation. They are hard to acquire from human fetal and primary tissues. Thus, further embodiments of the present disclosure provide methods for the generation, characterization and cryopreservation of human iPSC-derived microglia (iMGL) from HPCs, such as episomally reprogrammed iCell HPCs, under defined conditions. Cryopreserved iMGL retain purity, secrete immunomodulatory cytokines and phagocytose pHrodo Red labelled bacterial BioParticles and Amyloid βeta aggregates. The ability to produce essentially limitless quantities of iMGLs holds great promise for accelerating human neuroscience search into the role of microglia in normal and diseased states.

[0075] Further provided herein are microglia with disruption in TREM2, MeCP2, and/or SCNA. These microglia derived from patient derived iPSC provide an *in vitro* tool to create a more accurate model to understand complex interactions between human microglia, neurons, astrocytes in a 2D or 3D organoid systems and mimic neurogenerative diseases.

[0076] Also provided herein are methods of differentiating iPSCs to neural precursor cells (NPCs) without inhibition of SMAD signaling. These NPCs can be co-cultured with iPSC derived microglia to generate long term co-culture assays to mimic human brain development and complex intercellular interactions between neural lineages, microglia, endothelial cells, pericytes, and astrocytes in a dish derived from normal and/or disease-specific iPSC cells. The iPSCs may be maintained under hypoxic conditions before the onset of differentiation to generate NPCs. To initiate neural precursor differentiation, iPSCs may be seeded in the presence of a ROCK inhibitor or blebbistatin on an ECM-coated surface. The cells can be placed in media for the next 48 hours in the absence of a ROCK inhibitor. Next, the cells are pre-conditioned in DMEMF12 media supplemented with a GSK3 inhibitor for 72hours with a daily change in media under normoxic conditions. Cells can be harvested at the end of the preconditioning step and either replated back in a 2D format on ECM-coated plates or as 3D aggregates using Ultra low attachment plates or spinner flasks in the presence of a ROCK inhibitor or blebbistatin. The cultures may be fed every other day with E6 media supplemented with N2 for the next 8 days under normoxic conditions to produce NPCs. The different steps involved in the generation of NPCs are outlined in FIG. 45A.

[0077] The cells produced by the present methods may be used for disease modeling, drug discovery, and regenerative medicine. Also provided herein are methods of using the present cells (e.g., MSCs, endothelial cells, neural precursor cells and pericytes) for the production of a brain organoid or blood-brain barrier (BBB) model.

I. Definitions

[0078] As used herein the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one.

[0079] The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." As used herein "another" may mean at least a second or more.

[0080] The term "essentially" is to be understood that methods or compositions include only the specified steps or materials and those that do not materially affect the basic and novel characteristics of those methods and compositions.

[0081] As used herein, a composition or media that is "substantially free" of a specified substance or material contains ≤ 30%, ≤ 20%, ≤ 15%, more preferably ≤ 10%, even more preferably ≤ 5%, or most preferably ≤ 1% of the substance or material.

[0082] The terms "substantially" or "approximately" as used herein may be applied to modify any quantitative comparison, value, measurement, or other representation that could permissibly vary without resulting in a change in the basic function to which it is related.

[0083] The term "about" means, in general, within a standard deviation of the stated value as determined using a standard analytical technique for measuring the stated value. The terms can also be used by referring to plus or minus 5% of the stated value.

[0084] As used herein, "essentially free," in terms of a specified component, is used herein to mean that none of the specified component has been purposefully formulated into a composition and/or is present only as a contaminant or in trace amounts. The total amount of the specified component resulting from any unintended contamination of a composition is therefore well below 0.05%, preferably below 0.01%. Most preferred is a composition in which no amount of the specified component can be detected with standard analytical methods.

[0085] "Feeder-free" or "feeder-independent" is used herein to refer to a culture supplemented with cytokines and growth factors (*e.g.*, TGFβ, bFGF, LIF, analogs or mimetics thereof) as a replacement for the feeder cell layer. Thus, "feeder-free" or feeder-independent culture systems and media may be used to culture and maintain pluripotent cells in an undifferentiated and proliferative state. In some cases, feeder-free cultures utilize an animal-based matrix (*e.g.* MATRIGEL™) or are grown on a substrate such as fibronectin, collagen, or vitronectin. These approaches allow human stem cells to remain in an essentially undifferentiated state without the need for mouse fibroblast "feeder layers."

[0086] "Feeder layers" are defined herein as a coating layer of cells such as on the bottom of a culture dish. The feeder cells can release nutrients into the culture medium and provide a surface to which other cells, such as pluripotent stem cells, can attach.

[0087] The term "defined" or "fully-defined," when used in relation to a medium, an extracellular matrix, or a culture condition, refers to a medium, an extracellular matrix, or a culture condition in which the chemical composition and amounts of approximately all the components are known. For example, a defined medium does not contain undefined factors such as in fetal bovine serum, bovine serum albumin or human serum albumin. Generally, a defined medium comprises a basal media (*e.g.,* Dulbecco' s Modified Eagle' s Medium (DMEM), F12, or Roswell Park Memorial Institute Medium (RPMI) 1640, containing amino acids, vitamins, inorganic salts, buffers, antioxidants, and energy sources) which is supplemented with recombinant albumin, chemically defined lipids, and recombinant insulin. An example of a fully defined medium is Essential 8™ medium.

[0088] For a medium, extracellular matrix, or culture system used with human cells, the term "Xeno-Free (XF)" refers to a condition in which the materials used are not of non-human animal-origin.

[0089] "Treatment" or " treating" includes (1) inhibiting a disease in a subject or patient experiencing or displaying the pathology or symptomatology of the disease (*e.g.*, arresting further development of the pathology and/or symptomatology), (2) ameliorating a disease in a subject or patient that is experiencing or displaying the pathology or symptomatology of the disease (*e.g.*, reversing the pathology and/or symptomatology), and/or (3) effecting any measurable decrease in a disease in a subject or patient that is experiencing or displaying the pathology or symptomatology of the disease.

[0090] "Prophylactically treating" includes: (1) reducing or mitigating the risk of developing the disease in a subject or patient which may be at risk and/or predisposed to the disease but does not yet experience or display any or all of the pathology or symptomatology of the disease, and/or (2) slowing the onset of the pathology or symptomatology of a disease in a subject or patient which may be at risk and/or predisposed to the disease but does not yet experience or display any or all of the pathology or symptomatology of the disease.

[0091] As used herein, the term "patient" or "subject" refers to a living mammalian organism, such as a human, monkey, cow, sheep, goat, dog, cat, mouse, rat, guinea pig, or transgenic species thereof. In certain embodiments, the patient or subject is a primate. Non-limiting examples of human patients are adults, juveniles, infants and fetuses.

[0092] The term “effective,” as that term is used in the specification and/or claims, means adequate to accomplish a desired, expected, or intended result. “Effective amount,” “therapeutically effective amount” or “pharmaceutically effective amount” when used in the context of treating a patient or subject with a compound means that amount of the compound which, when administered to a subject or patient for treating or preventing a disease, is an amount sufficient to affect such treatment or prevention of the disease.

[0093] As generally used herein “pharmaceutically acceptable” refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues, organs, and/or bodily fluids of human beings and animals without excessive toxicity, irritation, allergic response, or other problems or complications commensurate with a reasonable benefit/risk ratio.

[0094] "Induced pluripotent stem cells (iPSCs)" are cells generated by reprogramming a somatic cell by expressing or inducing expression of a combination of factors (herein referred to as reprogramming factors). iPSCs can be generated using fetal, postnatal, newborn, juvenile, or adult somatic cells. In certain embodiments, factors that can be used to reprogram somatic cells to pluripotent stem cells include, for example, Oct4 (sometimes referred to as Oct 3/4), Sox2, c-Myc, Klf4, Nanog, and Lin28. In some embodiments, somatic cells are reprogrammed by expressing at least two reprogramming factors, at least three reprogramming factors, or four reprogramming factors to reprogram a somatic cell to a pluripotent stem cell.

[0095] The term “extracellular matrix protein” refers to a molecule which provides structural and biochemical support to the surrounding cells. The extracellular matrix protein can be recombinant and also refers to fragments or peptides thereof. Examples include collagen and heparin sulfate.

[0096] A “three-dimensional (3-D) culture” refers to an artificially-created environment in which biological cells are permitted to grow or interact with their surroundings in all three dimensions. The 3-D culture can be grown in various cell culture containers such as bioreactors, small capsules in which cells can grow into spheroids, or non-adherent culture plates. In particular aspects, the 3-D culture is scaffold-free. In contrast, a “two-dimensional (2-D)” culture refers to a cell culture such as a monolayer on an adherent surface.

[0097] As used herein, a "disruption" of a gene refers to the elimination or reduction of expression of one or more gene products encoded by the subject gene in a cell, compared to the level of expression of the gene product in the absence of the disruption. Exemplary gene products include mRNA and protein products encoded by the gene. Disruption in some cases is transient or reversible and in other cases is permanent. Disruption in some cases is of a functional or full-length protein or mRNA, despite the fact that a truncated or non-functional product may be produced. In some embodiments herein, gene activity or function, as opposed to expression, is disrupted. Gene disruption is generally induced by artificial methods, *i.e.*, by addition or introduction of a compound, molecule, complex, or composition, and/or by disruption of nucleic acid of or associated with the gene, such as at the DNA level. Exemplary methods for gene disruption include gene silencing, knockdown, knockout, and/or gene disruption techniques, such as gene editing. Examples include antisense technology, such as RNAi, siRNA, shRNA, and/or ribozymes, which generally result in transient reduction of expression, as well as gene editing techniques which result in targeted gene inactivation or disruption, *e.g.*, by induction of breaks and/or homologous recombination. Examples include insertions, mutations, and deletions. The disruptions typically result in the repression and/or complete absence of expression of a normal or "wild type" product encoded by the gene. Exemplary of such gene disruptions are insertions, frameshift and missense mutations, deletions, knock-in, and knock-out of the gene or part of the gene, including deletions of the entire gene. Such disruptions can occur in the coding region, *e.g.*, in one or more exons, resulting in the inability to produce a full-length product, functional product, or any product, such as by insertion of a stop codon. Such disruptions may also occur by disruptions in the promoter or enhancer or other region affecting activation of transcription, so as to prevent transcription of the gene. Gene disruptions include gene targeting, including targeted gene inactivation by homologous recombination.

II. iPSC Differentiation Methods

A. HPCs

[0098] The iPSCs can be differentiated into HPCs by methods known in the art such as described in U.S. Patent No. 8,372,642, which is incorporated by reference herein. In one method, combinations of BMP4, VEGF, Flt3 ligand, IL-3, and GM-CSF may be used to promote hematopoietic differentiation. In certain embodiments, the sequential exposure of cell cultures to a first media to prepare iPSCs for differentiation, a second media that includes

BMP4, VEGF, and FGF, followed by culture in a third media that includes Flt3 ligand, SCF, TPO, IL-3, and IL-6 can differentiate pluripotent cells into HPCs and hematopoietic cells. The second defined media can also comprise heparin. Further, inclusion of FGF-2 (50 ng/ml) in the media containing BMP4 and VEGF can enhance the efficiency of the generation of hematopoietic precursor cells from pluripotent cells. In addition, inclusion of a Glycogen synthase kinase 3 (GSK3) inhibitor (*e.g.,* CHIR99021, BIO, and SB-216763) in the first defined media can further enhance the production of HPCs.

[0099] Generally, differentiation of pluripotent cells into hematopoietic precursor cells may be performed using defined or undefined conditions. It will be appreciated that defined conditions are generally preferable in embodiments where the resulting cells are intended to be administered to a human subject. Hematopoietic stem cells may be derived from pluripotent stem cells under defined conditions (*e.g.*, using a TeSR media), and hematopoietic cells may be generated from embryoid bodies derived from pluripotent cells. In other embodiments, pluripotent cells may be co-cultured on OP9 cells or mouse embryonic fibroblast cells and subsequently differentiated.

[00100] Pluripotent cells may be allowed to form embryoid bodies or aggregates as a part of the differentiation process. The formation of "embryoid bodies" (EBs), or clusters of growing cells, in order to induce differentiation generally involves *in vitro* aggregation of human pluripotent stem cells into EBs and allows for the spontaneous and random differentiation of human pluripotent stem cells into multiple tissue types that represent endoderm, ectoderm, and mesoderm origins. Three-dimensional EBs can thus be used to produce some fraction of hematopoietic cells and endothelial cells.

[00101] To promote aggregate formation, the cells may be transferred to low-attachment plates for an overnight incubation in serum–free differentiation (SFD) medium, consisting of 75% IMDM (Gibco), 25% Ham's Modified F12 (Cellgro) supplemented with 0.05% N2 and 1% B-27 without RA supplements, 200 mM 1-glutamine, 0.05 mg/ml Ascorbic Acid-2-phosphate Magnesium Salt (Asc 2-P) (WAKO), and 4.5 x 10^{-4} MTG. The next day the cells may be collected from each well and centrifuged. The cells may then be resuspended in "EB differentiation media," which consists of SFD basal media supplemented with about 50 ng/ml bone morphogenetic factor (BMP4), about 50 ng/ml vascular endothelial growth factor (VEGF), and 50 ng/ml zb FGF for the first four days of differentiation. The cells are half fed every 48 hrs. On the fifth day of differentiation the media is replaced with a second media comprised of SFD media supplemented with 50 ng/ml stem cell factor (SCF), about 50 ng/ml Flt-3 ligand (Flt-3L), 50 ng/ml interleukin-6 (IL-6), 50 ng/ml interleukin-3 (IL-3), 50 ng/ml thrombopoieitin (TPO). The cells are half fed every 48 hrs with fresh differentiation media. The media changes are performed by spinning down the differentiation cultures at 300 g for 5 minutes and aspirating half the volume from the differentiating cultures and replenishing it with fresh media. In certain embodiments, the EB differentiation media may include about BMP4 (*e.g.*, about 50 ng/ml), VEGF (*e.g.*, about 50 ng/ml), and optionally FGF-2 (*e.g.*, about 25-75 ng/ml or about 50 ng/ml). The supernatant may be aspirated and replaced with fresh differentiation medium. Alternately the cells may be half fed every two days with fresh media. The cells may be harvested at different time points during the differentiation process.

[00102] HPCs may be cultured from pluripotent stem cells using a defined medium. Methods for the differentiation of pluripotent cells into hematopoietic CD34$^+$ stem cells using a defined media are described, *e.g.*, in U.S. Application 12/715,136 which is incorporated by reference in its entirety. It is anticipated that these methods may be used with the present disclosure.

[00103] For example, a defined medium may be used to induce hematopoietic CD34$^+$ differentiation. The defined medium may contain the growth factors BMP4, VEGF, Flt3 ligand, IL-3 and/or GMCSF. Pluripotent cells may be cultured in a first defined media comprising BMP4, VEGF, and optionally FGF-2, followed by culture in a second media comprising either (Flt3 ligand, IL-3, and GMCSF) or (Flt3 ligand, IL-3, IL-6, and TPO). The first and second media may also comprise one or more of SCF, IL-6, G-CSF, EPO, FGF-2, and/or TPO. Substantially hypoxic conditions (*e.g.*, less than 20% O2) may further promote hematopoietic or endothelial differentiation.

[00104] Cells may be substantially individualized via mechanical or enzymatic means (*e.g.*, using a trypsin or TrypLE^{TM}). A ROCK inhibitor (*e.g.*, H1152 or Y-27632) may also be included in the media. It is anticipated that these approaches may be automated using, *e.g.*, robotic automation.

[00105] In certain embodiments, substantially hypoxic conditions may be used to promote differentiation of pluripotent cells into hematopoietic progenitor cells. As would be recognized by one of skill in the art, an atmospheric oxygen content of less than about 20.8% would be considered hypoxic. Human cells in culture can grow in atmospheric conditions having reduced oxygen content as compared to ambient air. This relative hypoxia may be achieved by decreasing the atmospheric oxygen exposed to the culture media. Embryonic cells typically develop *in vivo* under reduced oxygen conditions, generally between about 1% and about 6% atmospheric oxygen, with carbon dioxide at ambient levels. Without wishing to be bound by theory, it is anticipated that hypoxic conditions may mimic an aspect of certain embryonic developmental conditions. As shown in the below examples, hypoxic conditions can be used in certain embodiments to promote additional differentiation of induced pluripotent cells into a more differentiated cell type, such as HPCs.

[00106] The following hypoxic conditions may be used to promote differentiation of pluripotent cells into hematopoietic progenitor cells. In certain embodiments, an atmospheric oxygen content of less than about 20%, less than about 19%, less than about 18%, less than about 17%, less than about 16%, less than about 15%, less than about 14%, less than about 13%, less than about 12%, less than about 11%, less than about 10%, less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, about 5%, about 4%, about 3%, about 2%, or about 1% may be used to promote differentiation into hematopoietic precursor cells. In certain embodiments, the hypoxic atmosphere comprises about 5% oxygen gas.

[00107] Regardless of the specific medium being used in any given hematopoietic progenitor cell expansion, the medium used is preferably supplemented with at least one cytokine at a concentration from about 0.1 ng/mL to about 500 ng mL, more usually 10 ng/mL to 100 ng/mL. Suitable cytokines include but are not limited to, c-kit ligand (KL) (also called steel factor (StI), mast cell growth factor (MGF), and stem cell factor (SCF)), IL-6, G-CSF, IL-3, GM-CSF, IL-1α, IL-11 MIP-1α, LIF, c-mpl ligand/TPO, and flk2/flk3 ligand (Flt2L or Flt3L). Particularly, the culture will include at least one of SCF, Flt3L and TPO. More particularly, the culture will include SCF, Flt3L and TPO.

[00108] In one embodiment, the cytokines are contained in the media and replenished by media perfusion. Alternatively, when using a bioreactor system, the cytokines may be added separately, without media perfusion, as a concentrated solution through separate inlet ports. When cytokines are added without perfusion, they will typically be added as a 10× to 100× solution in an amount equal to one-tenth to 1/100 of the volume in the bioreactors with fresh cytokines being added approximately every 2 to 4 days. Further, fresh concentrated cytokines also can be added separately in addition, to cytokines in the perfused media.

Exemplary HPC Differentiation Method

[00109] 2D HPC differentiation: iPSCs may be maintained on MATRIGEL™ or Vitronectin in the presence of E8 and adapted to hypoxia for at least 5-10 passages. Cells are split from sub confluent iPSCs and plated at a density of 0.25 million cells/well onto Amine culture dishes in the presence Serum Free Defined (SFD) media supplemented with 5 uM blebbistatin. 24 hrs post plating SFD media supplemented with 50 ng/ml of BMP4, VEGF and FGF2 is added to the culture. The following day, fresh media is exchanged to remove blebbistatin. On the fifth day of the differentiation process, the cells are placed in media containing 50 ng/ml Flt-3 Ligand, SCF, TP0, IL3 and IL6 with 5U/ml of heparin. The cells are fed every 48 hrs throughout the differentiation process. The entire process is performed under hypoxic conditions and on charged amine plates. HPCs are quantified by the presence of CD43/CD34 cells and CFU.

[00110] 3D HPC Differentiation: Cells were split from sub confluent iPSCs and plated at a density of 0.25-0.5 million cells per ml into a spinner flask in the presence of Serum Free Defined (SFD) media supplemented with 5 μM blebbistatin or 1 μM H1152. 24 hrs post plating SFD media supplemented with 50 ng/ml of BMP4, VEGF and FGF2 was exchanged. On the fifth day of the differentiation process the cells were placed in media containing 50 ng/ml Flt-3 Ligand, SCF, TP0, IL3 and IL6 with 5-10 U/ml of heparin. The cells were fed every 48 hrs throughout the differentiation process. The entire process was performed under hypoxic conditions. HPCs quantified by presence of CD43/CD34. HPCs are MACS sorted using CD34 beads.

B. Gene Disruption

[00111] In certain aspects, TREM2, MeCP2, and/or SCNA gene expression, activity or function is disrupted in cells, such as PSCs (*e.g.,* ESCs or iPSCs). In some embodiments, the gene disruption is carried out by effecting a disruption in the gene, such as a knock-out, insertion, missense or frameshift mutation, such as biallelic frameshift mutation, deletion of all or part of the gene, *e.g.,* one or more exon or portion therefore, and/or knock-in. For example, the disruption can be effected be sequence-specific or targeted nucleases, including DNA-binding targeted nucleases such as zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs), and RNA-guided nucleases such as a CRISPR-associated nuclease (Cas), specifically designed to be targeted to the sequence of the gene or a portion thereof.

[00112] In some embodiments, the disruption of the expression, activity, and/or function of the gene is carried out by disrupting the gene. In some aspects, the gene is disrupted so that its expression is reduced by at least at or about 20, 30, or 40%, generally at least at or about 50, 60, 70, 80, 90, or 95% as compared to the expression in the absence of the gene disruption or in the absence of the components introduced to effect the disruption.

[00113] In some embodiments, the disruption is transient or reversible, such that expression of the gene is restored at a later time. In other embodiments, the disruption is not reversible or transient, *e.g.*, is permanent.

[00114] In some embodiments, gene disruption is carried out by induction of one or more double-stranded breaks and/or one or more single-stranded breaks in the gene, typically in a targeted manner. In some embodiments, the double-stranded or single-stranded breaks are made by a nuclease, *e.g.*, an endonuclease, such as a gene-targeted nuclease. In some aspects, the breaks are induced in the coding region of the gene, *e.g.*, in an exon. For example, in some embodiments, the induction occurs near the N-terminal portion of the coding region, *e.g.*, in the first exon, in the second exon, or in a subsequent exon.

[00115] In some aspects, the double-stranded or single-stranded breaks undergo repair via a cellular repair process, such as by non-homologous end-joining (NHEJ) or homology-directed repair (HDR). In some aspects, the repair process is error-prone and results in disruption of the gene, such as a frameshift mutation, *e.g.*, biallelic frameshift mutation, which can result in complete knockout of the gene. For example, in some aspects, the disruption comprises inducing a deletion, mutation, and/or insertion. In some embodiments, the disruption results in the presence of an early stop codon. In some aspects, the presence of an insertion, deletion, translocation, frameshift mutation, and/or a premature stop codon results in disruption of the expression, activity, and/or function of the gene.

[00116] In some embodiments, gene disruption is achieved using antisense techniques, such as by RNA interference (RNAi), short interfering RNA (siRNA), short hairpin (shRNA), and/or ribozymes are used to selectively suppress or repress expression of the gene. siRNA technology is RNAi which employs a double-stranded RNA molecule having a sequence homologous with the nucleotide sequence of mRNA which is transcribed from the gene, and a sequence complementary with the nucleotide sequence. siRNA generally is homologous/complementary with one region of mRNA which is transcribed from the gene, or may be siRNA including a plurality of RNA molecules which are homologous/complementary with different regions. In some aspects, the siRNA is comprised in a polycistronic construct. In particular aspects, the siRNA suppresses both wild-type and mutant protein translation from endogenous mRNA.

[00117] In some embodiments, the disruption is achieved using a DNA-targeting molecule, such as a DNA-binding protein or DNA-binding nucleic acid, or complex, compound, or composition, containing the same, which specifically binds to or hybridizes to the gene. In some embodiments, the DNA-targeting molecule comprises a DNA-binding domain, e.g., a zinc finger protein (ZFP) DNA-binding domain, a transcription activator-like protein (TAL) or TAL effector (TALE) DNA-binding domain, a clustered regularly interspaced short palindromic repeats (CRISPR) DNA-binding domain, or a DNA-binding domain from a meganuclease. Zinc finger, TALE, and CRISPR system binding domains can be engineered to bind to a predetermined nucleotide sequence, for example via engineering (altering one or more amino acids) of the recognition helix region of a naturally occurring zinc finger or TALE protein. Engineered DNA binding proteins (zinc fingers or TALEs) are proteins that are non-naturally occurring. Rational criteria for design include application of substitution rules and computerized algorithms for processing information in a database storing information of existing ZFP and/or TALE designs and binding data. See, for example, U.S. Patent Nos. 6,140,081; 6,453,242; and 6,534,261; see also WO 98/53058; WO 98/53059; WO 98/53060; WO 02/016536 and WO 03/016496 and U.S. Publication No. 2011/0301073.

[00118] In some embodiments, the DNA-targeting molecule, complex, or combination contains a DNA-binding molecule and one or more additional domain, such as an effector domain to facilitate the repression or disruption of the gene. For example, in some embodiments, the gene disruption is carried out by fusion proteins that comprise DNA-binding proteins and a heterologous regulatory domain or functional fragment thereof. In some aspects, domains include, *e.g.*, transcription factor domains such as activators, repressors, co-activators, co-repressors, silencers, oncogenes, DNA repair enzymes and their associated factors and modifiers, DNA rearrangement enzymes and their associated factors and modifiers, chromatin associated proteins and their modifiers, *e.g.* kinases, acetylases and deacetylases, and DNA modifying enzymes, *e.g.* methyltransferases, topoisomerases, helicases, ligases, kinases, phosphatases, polymerases, endonucleases, and their associated factors and modifiers. See, for example, U.S. Patent Application Publication Nos. 2005/0064474; 2006/0188987 and

2007/0218528, incorporated by reference in their entireties herein, for details regarding fusions of DNA-binding domains and nuclease cleavage domains. In some aspects, the additional domain is a nuclease domain. Thus, in some embodiments, gene disruption is facilitated by gene or genome editing, using engineered proteins, such as nucleases and nuclease-containing complexes or fusion proteins, composed of sequence-specific DNA-binding domains fused to or complexed with non-specific DNA-cleavage molecules such as nucleases.

[00119] In some aspects, these targeted chimeric nucleases or nuclease-containing complexes carry out precise genetic modifications by inducing targeted double-stranded breaks or single-stranded breaks, stimulating the cellular DNA-repair mechanisms, including error-prone nonhomologous end joining (NHEJ) and homology-directed repair (HDR). In some embodiments the nuclease is an endonuclease, such as a zinc finger nuclease (ZFN), TALE nuclease (TALEN), and RNA-guided endonuclease (RGEN), such as a CRISPR-associated (Cas) protein, or a meganuclease.

[00120] In some embodiments, a donor nucleic acid, *e.g.*, a donor plasmid or nucleic acid encoding the genetically engineered antigen receptor, is provided and is inserted by HDR at the site of gene editing following the introduction of the DSBs. Thus, in some embodiments, the disruption of the gene and the introduction of the antigen receptor, *e.g.,* CAR, are carried out simultaneously, whereby the gene is disrupted in part by knock-in or insertion of the CAR-encoding nucleic acid.

[00121] In some embodiments, no donor nucleic acid is provided. In some aspects, NHEJ-mediated repair following introduction of DSBs results in insertion or deletion mutations that can cause gene disruption, *e.g.*, by creating missense mutations or frameshifts.

1. ZFPs and ZFNs

[00122] In some embodiments, the DNA-targeting molecule includes a DNA-binding protein such as one or more zinc finger protein (ZFP) or transcription activator-like protein (TAL), fused to an effector protein such as an endonuclease. Examples include ZFNs, TALEs, and TALENs.

[00123] In some embodiments, the DNA-targeting molecule comprises one or more zinc-finger proteins (ZFPs) or domains thereof that bind to DNA in a sequence-specific manner. A ZFP or domain thereof is a protein or domain within a larger protein that binds DNA in a sequence-specific manner through one or more zinc fingers, regions of amino acid sequence within the binding domain whose structure is stabilized through coordination of a zinc ion. The term zinc finger DNA binding protein is often abbreviated as zinc finger protein or ZFP. Among the ZFPs are artificial ZFP domains targeting specific DNA sequences, typically 9-18 nucleotides long, generated by assembly of individual fingers.

[00124] ZFPs include those in which a single finger domain is approximately 30 amino acids in length and contains an alpha helix containing two invariant histidine residues coordinated through zinc with two cysteines of a single beta turn, and having two, three, four, five, or six fingers. Generally, sequence-specificity of a ZFP may be altered by making amino acid substitutions at the four helix positions (-1, 2, 3 and 6) on a zinc finger recognition helix. Thus, in some embodiments, the ZFP or ZFP-containing molecule is non-naturally occurring, *e.g.*, is engineered to bind to a target site of choice.

[00125] In some aspects, disruption of MeCP2 is carried out by contacting a first target site in the gene with a first ZFP, thereby disrupting the gene. In some embodiments, the target site in the gene is contacted with a fusion ZFP comprising six fingers and the regulatory domain, thereby inhibiting expression of the gene.

[00126] In some embodiments, the step of contacting further comprises contacting a second target site in the gene with a second ZFP. In some aspects, the first and second target sites are adjacent. In some embodiments, the first and second ZFPs are covalently linked. In some aspects, the first ZFP is a fusion protein comprising a regulatory domain or at least two regulatory domains.

[00127] In some embodiments, the first and second ZFPs are fusion proteins, each comprising a regulatory domain or each comprising at least two regulatory domains. In some embodiments, the regulatory domain is a transcriptional repressor, a transcriptional activator, an endonuclease, a methyl transferase, a histone acetyltransferase, or a histone deacetylase.

[00128] In some embodiments, the ZFP is encoded by a ZFP nucleic acid operably linked to a promoter. In some aspects, the method further comprises the step of first administering the nucleic acid to the cell in a lipid:nucleic acid complex or as naked nucleic acid. In some embodiments, the ZFP is encoded by an expression vector comprising a ZFP nucleic acid operably linked to a promoter. In some embodiments, the ZFP is encoded by a nucleic acid operably linked to an inducible promoter. In some aspects, the ZFP is encoded by a nucleic acid operably linked to a weak promoter.

[00129] In some embodiments, the target site is upstream of a transcription initiation site of the gene. In some aspects, the target site is adjacent to a transcription initiation site of the gene. In some aspects, the target site is adjacent to an RNA polymerase pause site downstream of a transcription initiation site of the gene.

[00130] In some embodiments, the DNA-targeting molecule is or comprises a zinc-finger DNA binding domain fused to a DNA cleavage domain to form a zinc-finger nuclease (ZFN). In some embodiments, fusion proteins comprise the cleavage domain (or cleavage half-domain) from at least one Type liS restriction enzyme and one or more zinc finger binding domains, which may or may not be engineered. In some embodiments, the cleavage domain is from the Type liS restriction endonuclease Fok I. Fok I generally catalyzes double-stranded cleavage of DNA, at 9 nucleotides from its recognition site on one strand and 13 nucleotides from its recognition site on the other.

[00131] In some embodiments, ZFNs target a gene present in the engineered cell. In some aspects, the ZFNs efficiently generate a double strand break (DSB), for example at a predetermined site in the coding region of the gene. Typical regions targeted include exons, regions encoding N terminal regions, first exon, second exon, and promoter or enhancer regions. In some embodiments, transient expression of the ZFNs promotes highly efficient and permanent disruption of the target gene in the engineered cells. In particular, in some embodiments, delivery of the ZFNs results in the permanent disruption of the gene with efficiencies surpassing 50%.

[00132] Many gene-specific engineered zinc fingers are available commercially. For example, Sangamo Biosciences (Richmond, CA, USA) has developed a platform (CompoZr) for zinc-finger construction in partnership with Sigma-Aldrich (St. Louis, MO, USA), allowing investigators to bypass zinc-finger construction and validation altogether, and provides specifically targeted zinc fingers for thousands of proteins (Gaj et al., Trends in Biotechnology, 2013, 31(7), 397-405). In some embodiments, commercially available zinc fingers are used or are custom designed.

2. TALs, TALEs and TALENs

[00133] In some embodiments, the DNA-targeting molecule comprises a naturally occurring or engineered (non-naturally occurring) transcription activator-like protein (TAL) DNA binding domain, such as in a transcription activator-like protein effector (TALE) protein, See, e.g., U.S. Patent Publication No. 2011/0301073, incorporated by reference in its entirety herein.

[00134] A TALE DNA binding domain or TALE is a polypeptide comprising one or more TALE repeat domains/units. The repeat domains are involved in binding of the TALE to its cognate target DNA sequence. A single "repeat unit" (also referred to as a "repeat") is typically 33-35 amino acids in length and exhibits at least some sequence homology with other TALE repeat sequences within a naturally occurring TALE protein. Each TALE repeat unit includes 1 or 2 DNA-binding residues making up the Repeat Variable Diresidue (RVD), typically at positions 12 and/or 13 of the repeat. The natural (canonical) code for DNA recognition of these TALEs has been determined such that an HD sequence at positions 12 and 13 leads to a binding to cytosine (C), NG binds to T, NI to A, NN binds to G or A, and NO binds to T and non-canonical (atypical) RVDs are also known. See, U.S. Patent Publication No. 2011/0301073. In some embodiments, TALEs may be targeted to any gene by design of TAL arrays with specificity to the target DNA sequence. The target sequence generally begins with a thymidine.

[00135] In some embodiments, the molecule is a DNA binding endonuclease, such as a TALE nuclease (TALEN). In some aspects the TALEN is a fusion protein comprising a DNA-binding domain derived from a TALE and a nuclease catalytic domain to cleave a nucleic acid target sequence.

[00136] In some embodiments, the TALEN recognizes and cleaves the target sequence in the gene. In some aspects, cleavage of the DNA results in double-stranded breaks. In some aspects the breaks stimulate the rate of homologous recombination or non-homologous end joining (NHEJ). Generally, NHEJ is an imperfect repair process that often results in changes to the DNA sequence at the site of the cleavage. In some aspects, repair mechanisms involve rejoining of what remains of the two DNA ends through direct re-ligation (Critchlow and Jackson, 1998) or via the so-called microhomology-mediated end joining. In some embodiments, repair via NHEJ results in small insertions or deletions and can be used to disrupt and thereby repress the gene. In some embodiments, the modification may be a substitution, deletion, or addition of at least one nucleotide. In some aspects, cells in which a cleavage-induced mutagenesis event, i.e. a mutagenesis event consecutive to an NHEJ event, has occurred can be identified and/or selected by well-known methods in the art.

[00137] In some embodiments, TALE repeats are assembled to specifically target a gene. A library of TALENs targeting 18,740 human protein-coding genes has been constructed. Custom-designed TALE arrays are commercially available through Cellectis Bioresearch (Paris, France), Transposagen Biopharmaceuticals (Lexington, KY, USA), and Life Technologies (Grand Island, NY, USA).

[00138] In some embodiments the TALENs are introduced as trans genes encoded by one or more plasmid vectors. In some aspects, the plasmid vector can contain a selection marker which provides for identification and/or selection of cells which received said vector.

3. RGENs (CRISPR/Cas systems)

[00139] In some embodiments, the disruption is carried out using one or more DNA-binding nucleic acids, such as disruption via an RNA-guided endonuclease (RGEN). For example, the disruption can be carried out using clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins. In general, "CRISPR system" refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated ("Cas") genes, including sequences encoding a Cas gene, a tracr (trans-activating CRISPR) sequence (*e.g.* tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a "direct repeat" and a tracrRNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a "spacer" in the context of an endogenous CRISPR system), and/or other sequences and transcripts from a CRISPR locus.

[00140] The CRISPR/Cas nuclease or CRISPR/Cas nuclease system can include a non-coding RNA molecule (guide) RNA, which sequence-specifically binds to DNA, and a Cas protein (*e.g.*, Cas9), with nuclease functionality (*e.g.*, two nuclease domains). One or more elements of a CRISPR system can derive from a type I, type II, or type III CRISPR system, *e.g.,* derived from a particular organism comprising an endogenous CRISPR system, such as Streptococcus pyogenes.

[00141] In some aspects, a Cas nuclease and gRNA (including a fusion of crRNA specific for the target sequence and fixed tracrRNA) are introduced into the cell. In general, target sites at the 5' end of the gRNA target the Cas nuclease to the target site, *e.g.*, the gene, using complementary base pairing. The target site may be selected based on its location immediately 5' of a protospacer adjacent motif (PAM) sequence, such as typically NGG, or NAG. In this respect, the gRNA is targeted to the desired sequence by modifying the first 20, 19, 18, 17, 16, 15, 14, 14, 12, 11, or 10 nucleotides of the guide RNA to correspond to the target DNA sequence. In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence. Typically, "target sequence" generally refers to a sequence to which a guide sequence is designed to have complementarity, where hybridization between the target sequence and a guide sequence promotes the formation of a CRISPR complex. Full complementarity is not necessarily required, provided there is sufficient complementarity to cause hybridization and promote formation of a CRISPR complex.

[00142] The CRISPR system can induce double stranded breaks (DSBs) at the target site, followed by disruptions as discussed herein. In other embodiments, Cas9 variants, deemed "nickases," are used to nick a single strand at the target site. Paired nickases can be used, *e.g.*, to improve specificity, each directed by a pair of different gRNAs targeting sequences such that upon introduction of the nicks simultaneously, a 5' overhang is introduced. In other embodiments, catalytically inactive Cas9 is fused to a heterologous effector domain such as a transcriptional repressor or activator, to affect gene expression.

[00143] The target sequence may comprise any polynucleotide, such as DNA or RNA polynucleotides. The target sequence may be located in the nucleus or cytoplasm of the cell, such as within an organelle of the cell. Generally, a sequence or template that may be used for recombination into the targeted locus comprising the target sequences is referred to as an "editing template" or "editing polynucleotide" or "editing sequence". In some aspects, an exogenous template polynucleotide may be referred to as an editing template. In some aspects, the recombination is homologous recombination.

[00144] Typically, in the context of an endogenous CRISPR system, formation of the CRISPR complex (comprising the guide sequence hybridized to the target sequence and complexed with one or more Cas proteins) results in cleavage of one or both strands in or near (*e.g.* within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or more base pairs from) the target sequence. The tracr sequence, which may comprise or consist of all or a portion of a wild-type tracr sequence (*e.g.* about or more than about 20, 26, 32, 45, 48, 54, 63, 67, 85, or more nucleotides of a wild-type tracr sequence), may also form part of the CRISPR complex, such as by hybridization along at least a portion of the tracr sequence to all or a portion of a tracr mate sequence that is operably linked to the guide sequence. The tracr sequence has sufficient complementarity to a tracr mate sequence to hybridize and participate in formation of the CRISPR complex, such as at least 50%, 60%, 70%, 80%, 90%, 95% or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned.

[00145] One or more vectors driving expression of one or more elements of the CRISPR system can be introduced into the cell such that expression of the elements of the CRISPR system direct formation of the CRISPR complex at one or more target sites. Components can also be delivered to cells as proteins and/or RNA. For example, a Cas enzyme, a guide sequence linked to a tracr-mate sequence, and a tracr sequence could each be operably linked to separate regulatory elements on separate vectors. Alternatively, two or more of the elements expressed from the same or different regulatory elements, may be combined in a single vector, with one or more additional vectors providing any components of the CRISPR system not included in the first vector. The vector may comprise one or more insertion sites, such as a restriction endonuclease recognition sequence (also referred to as a "cloning site"). In some embodiments, one or more insertion sites are located upstream and/or downstream of one or more sequence elements of one or more vectors. When multiple different guide sequences are used, a single expression construct may be used to target CRISPR activity to multiple different, corresponding target sequences within a cell.

[00146] A vector may comprise a regulatory element operably linked to an enzyme-coding sequence encoding the CRISPR enzyme, such as a Cas protein. Non-limiting examples of Cas proteins include Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known as Csn1 and Csx12), Cas10, Csy1, Csy2, Csy3, Cse1, Cse2, Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, homologs thereof, or modified versions thereof. These enzymes are known; for example, the amino acid sequence of S. pyogenes Cas9 protein may be found in the SwissProt database under accession number Q99ZW2.

[00147] The CRISPR enzyme can be Cas9 (*e.g.,* from S. pyogenes or S. pneumonia). The CRISPR enzyme can direct cleavage of one or both strands at the location of a target sequence, such as within the target sequence and/or within the complement of the target sequence. The vector can encode a CRISPR enzyme that is mutated with respect to a corresponding wild-type enzyme such that the mutated CRISPR enzyme lacks the ability to cleave one or both strands of a target polynucleotide containing a target sequence. For example, an aspartate-to-alanine substitution (D10A) in the RuvC I catalytic domain of Cas9 from S. pyogenes converts Cas9 from a nuclease that cleaves both strands to a nickase (cleaves a single strand). In some embodiments, a Cas9 nickase may be used in combination with guide sequence(s), *e.g.*, two guide sequences, which target respectively sense and antisense strands of the DNA target. This combination allows both strands to be nicked and used to induce NHEJ or HDR.

[00148] In some embodiments, an enzyme coding sequence encoding the CRISPR enzyme is codon optimized for expression in particular cells, such as eukaryotic cells. The eukaryotic cells may be those of or derived from a particular organism, such as a mammal, including but not limited to human, mouse, rat, rabbit, dog, or non-human primate. In general, codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Various species exhibit particular bias for certain codons of a particular amino acid. Codon bias (differences in codon usage between organisms) often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, among other things, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization.

[00149] In general, a guide sequence is any polynucleotide sequence having sufficient complementarity with a target polynucleotide sequence to hybridize with the target sequence and direct sequence-specific binding of the CRISPR complex to the target sequence. In some embodiments, the degree of complementarity between a guide sequence and its corresponding target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more.

[00150] Optimal alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting example of which include the Smith-Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows-Wheeler Transform (*e.g.* the Burrows Wheeler Aligner), Clustal W, Clustal X, BLAT, Novoalign (Novocraft Technologies, ELAND (Illumina, San Diego, Calif.), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net).

[00151] The CRISPR enzyme may be part of a fusion protein comprising one or more heterologous protein domains. A CRISPR enzyme fusion protein may comprise any additional protein sequence, and optionally a linker sequence between any two domains. Examples of protein domains that may be fused to a CRISPR enzyme include, without limitation, epitope tags, reporter gene sequences, and protein domains having one or more of the following activities: methylase activity, demethylase activity, transcription activation activity, transcription repression activity, transcription release factor activity, histone modification activity, RNA cleavage activity and nucleic acid binding activity. Non-limiting examples of epitope tags include histidine (His) tags, V5 tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Examples of reporter genes include, but are not limited to, glutathione-5-transferase (GST), horseradish peroxidase (HRP), chloramphenicol acetyltransferase (CAT) beta galactosidase, beta-glucuronidase, luciferase, green fluorescent protein (GFP), HcRed, DsRed, cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and autofluorescent proteins including blue fluorescent protein (BFP). A CRISPR enzyme may be fused to a gene sequence encoding a protein or a fragment of a protein that bind DNA molecules or bind other cellular molecules, including but not limited to maltose binding protein (MBP), S-tag, Lex A DNA binding domain (DBD) fusions, GAL4A DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions.

C. Charged Cell Surfaces

[00152] In some embodiments, the present disclosure concerns charged surfaces for cell culture. The charged surface may be positively charged, such as an amine surface or nitrogen-containing functional groups, or negatively charged, such as a carboxyl surface or oxygen-containing functional groups. The cell surfaces may be treated to alter the surface charge of the culture vessel.

[00153] In some aspects, the surface is neutrally charged, such as a surface comprising both negatively charged and positively charged functional groups. For example, the CORNING PRIMARIA® surface features a unique mixture of oxygen-containing (negatively charged) and nitrogen-containing (positive charged) functional groups on the polystyrene surface. The surface supports the growth of cells that can exhibit poor attachment or limited differentiation potential when cultured on traditional TC surfaces. In some aspects, the surface comprises a ULA surface coating. For example, the corning ultra-low attachment surface is a covalently bound hydrogel layer that is hydrophilic and neutrally charged. Since proteins and other biomolecules passively adsorb to polystyrene surfaces through either hydrophobic or ionic interactions, this hydrogel naturally inhibits nonspecific immobilization via these forces, thus inhibiting subsequent cell attachment. This surface is very stable, noncytotoxic, biologically inert and nondegradable. Other examples that could support the generation of microglia from HPCs include: Corning CellBIND culture (U.S. Patent 6,617,152) uses a higher energy microwave plasma to incorporate more oxygen onto the polystyrene surface rendering it more hydrophilic (wettable) while increasing the stability of the surface compared with traditional plasma or corona discharge treated surfaces. Corning Synthemax self-coating substrate is a unique, animal-free, synthetic Vitronectin-based peptide containing the RGD motif and flanking sequences. The synthetic peptides are covalently bound to a polymer backbone for passive coating, orienting, and presenting the peptide for optimal cell binding and signaling.

[00154] The cell culture surface may be coated with a plasma polymerized film. The source of the plasma polymerization is one or more monomers. Useful polymerizable monomers may include unsaturated organic compounds such as olefinic amines, halogenated olefins, olefinic carboxylic acids and carboxylates, olefinic nitrile compounds, oxygenated olefins and olefinic hydrocarbons. In some embodiments, the olefins may include vinylic and allylic forms. In other embodiments, cyclic compounds such as cyclohexane, cyclopentane and cyclopropane may be used.

[00155] As will be recognized by those skilled in the art, various plasma polymerization techniques may be utilized to deposit the one or more monomers onto the cell culture surfaces. Preferably, a positively charged polymerized film is deposited on the surfaces. As will be appreciated by one skilled in the art, the plasma polymerized surface may have a negative charge depending on the proteins to be used therewith. Amine is preferably used as the monomer source of the polymer. In some embodiments, the plasma polymerized monomer is made using plasma sources to generate a gas discharge that provides energy to initiate polymerization of gaseous monomers and allows a thin polymer film to deposit on a culture vessel. Cyclic compounds may be utilized which may include gas plasmas by glow discharge methods. Derivatives of these cyclic compounds, such as 1,2- diaminocyclohexane for instance, are also commonly polymerizable in gas plasmas.

[00156] Mixtures of polymerizable monomers may be used. Additionally, polymerizable monomers may be blended with other gases not generally considered as polymerizable in themselves, examples being argon, nitrogen and hydrogen.

[00157] It is contemplated that any culture vessel that is useful for adherent cultures may be used. Preferred cell culture vessel configurations contemplated by the present disclosure include multiwell plates (such as 6-well, 12-well and 24-well plates), dishes (such as petri dishes), test tubes, culture flasks, roller bottles, tube or shaker flasks, and the like.

[00158] Material for the cell culture surface may include plastic (e.g. polystyrene, acrylonitrile butadiene styrene, polycarbonate); glass; microporous filters (e.g., cellulose, nylon, glass fiber, polyester, and polycarbonate); materials for bio-reactors used in batch or continuous cell culture or in genetic engineering (e.g., bioreactors), which may include hollow fiber tubes or micro carrier beads; polytetrafluoroethylene (Teflon®), ceramics and related polymeric materials.

[00159] In particular aspects, the cell culture is free of or essentially free of any extracellular matrix proteins, such as laminin, fibronectin, vitronectin, MATRIGEL™, tenascin, entactin, thrombospondin, elastin, gelatin, collagen, fibrillin, merosin, anchorin, chondronectin, link protein, bone sialoprotein, osteocalcin, osteopontin, epinectin, hyaluronectin, undulin, epiligrin, and kalinin.

D. Differentiation of HPCs to Microglia

[00160] Microglia are innate immune cells of the central nervous system that perform critical roles in brain development, homeostasis, and immune regulation. They are hard to acquire from human fetal and primary tissues. In certain embodiments, the present methods describe the generation, characterization and cryopreservation of human iPSC-derived microglia (iMGL) from episomally reprogrammed HPCs under defined conditions.

Cryopreserved iMGL retain purity, secrete immunomodulatory cytokines and phagocytose pHrodo Red labelled bacterial BioParticles and Amyloid βeta aggregates. The ability to produce essentially limitless quantities of iMGLs holds great promise for accelerating human neuroscience research into the role of microglia in normal and diseased states.

[00161] In an exemplary method, fresh or cryopreserved HPCs are thawed and plated in Microglia Differentiation Media comprising FLT-3 ligand and IL-3. The cells may be plated at a density of 10-50 K/cm2, such as 20-35K/cm2. The Microglia Differentiation Medium may comprise IL-34, TGFβ1, or M-CSF (MDM) or the respective analogs or mimetics thereof. The culturing may be performed on MATRIGEL™ coated plate or a charged surface such as a Primaria plate or Ultra low attachment plate or a tissue culture plate (TC) or a non-tissue culture plate (Non-TC) and may be high-throughput, such as a 96 well plate (e.g., 200 μl Microglia Differentiation Medium per well). The cells may be half fed every 48 hrs with 50μl media per well of 2X Microglia Differentiation media (MDM) the next 23 days of differentiation. In specific aspects, the differentiation is performed in the absence of ECM proteins, such as MATRIGEL®. The cells are harvested with cold PBS on day 23 and the total viable cell number is quantified using an automated cell counter. The cells are stained for surface expression of CD11b, CD11c, CD45, CD33, TREM-2 and intracellular expression of TREM-2, IBA, CX3CR1, P2RY12 and TMEM119.

E. Endothelial Cells

[00162] iPSCs maintained on MATRIGEL™ or Vitronectin in the presence of E8, may be adapted to hypoxia for at least 5-10 passages. Cells may be split from sub confluent iPSCs and plated at a density of 0.25 million cells/well onto Amine culture dishes in the presence Serum Free Defined (SFD) media supplemented with 5 uM blebbistatin or 1 uM H1152. 24 hrs post plating SFD media supplemented with 50 ng/ml of BMP4, VEGF and FGF2 may be added to the culture. The cells may be fed every 48 hrs throughout the differentiation process. The entire process may be performed under hypoxic conditions. The cells harvested at the end of differentiation can be cryopreserved or replated on a carboxyl surface at a density of 25k/cm2 to initiate endothelial differentiation in the presence of VascuLife VEGF Endothelial Medium or SFD Endothelial Medium.

[00163] In an exemplary method, cryopreserved day 6 HPCs or live cultures are plated at 25k/cm^2 on a carboxyl surface in the presence of VascuLife VEGF Endothelial Medium or SFD Endothelial Medium and hypoxic conditions. The cells are given a fresh feed of endothelial medium 24 hrs post plating and the cultures are fed every 48 hours until they reached confluency. It may take 5-6 days for cells to reach confluency. The cells are harvested using TrypLE Select, stained for surface endothelial markers CD31, CD105 and CD144 and replated onto a carboxyl surface at 25k/cm^2 with endothelial medium and placed in hypoxic incubator conditions. The cells are given a full feed of endothelial medium on days 2, 4, 6 post-split. On day 7, the cells are harvested, stained, and replated in the same manner three more times.

[00164] In some embodiments, the endothelial cells are converted to Brain Microvascular Endothelial cells. In an exemplary method, live or cryopreserved HPCs (e.g., day 7 HPCs derived on Amine surface in the presence of SFD supplemented with BMP4, VEGF and FGF2) are plated onto a ECM containing fibronectin (e.g., 50-200 µg/mL, particularly 100 µg/mL) and Collagen I (e.g., 100-500 µg/mL, particularly 400 µg/mL) with ECRA Medium (Human Endothelial SFM [Gibco], 1% Platelet-poor plasma-derived bovine serum [Fisher], 20ng/mL bFGF [Promega], 10uM Retinoic Acid). The cells may be plated at a density of 50-100 k/cm^2, particularly 75 k/cm^2. The cultures can be maintained under hypoxic incubator conditions. The cultures may be fed with ECRA Medium every other day until confluent. Confluent cultures are then harvested, such as by using TrypLE. Staining may be performed on harvested cells to detect PECAM-1 (CD31) and GLUT-1. Harvested cells may be replated, such as on Transwell inserts with ECRA Medium and placed in hypoxic incubator conditions. The culture may be fed ECRA Medium every other day until confluent. Confluent cultures may be tested for transendothelial electrical resistance (TEER).

F. Mesenchymal Cells

[00165] In some embodiments, the iPSCs are differentiated to MSCs. For example, FIG. 5C shows a schematic representation of the 2D HPC differentiation process to generate MSCs. iPSCs maintained on MATRIGEL™ or Vitronectin in the presence of E8, are adapted to hypoxia for at least 5-10 passages. Cells are split from sub confluent iPSCs and plated at a density of 0.25 million cells/well onto amine culture dishes in the presence Serum Free Defined (SFD) media supplemented with 5 uM blebbistatin or 10 uM H1152. 24 hrs post plating SFD media supplemented with 50ng/ml of BMP4, VEGF and FGF2 is added to the culture. The cells are fed every 48 hrs throughout the differentiation process. The entire process is performed under hypoxic conditions. At the end of day 6 or 7 of differentiation the cells are placed in GMP –MSC media. The cells are allowed to grow to confluency and harvested at the end of each passage, then replated on an amine surface at a density of 50K/cm^2 in GMP-MSC Media supplemented with 5 uM blebbistatin or 10 uM H1152 to selectively allow the growth and proliferation of MSCs.

[00166] In some aspects, cryopreserved day 6 HPCs or live cultures at the end of day 6 differentiation are placed in the presence of MSC media in the presence of 10 uM H1152 on an Amine charged surface plates. The cells are given a fresh feed of MSC media 24 hrs post plating and the cultures were fed every 48 hours until they reached confluency. It took 5-6 days for cells to reach confluency. The cells are harvested using TrypLE, and stained for surface MSC markers CD73, CD44, CD105, CD49d, and the absence of Endothelial markers CD31 and CD144. The emerging cultures are passaged three times using the process described above under hypoxic conditions and amine surface. The cultures are transitioned to normoxia and normal tissue culture plates at P4.

[00167] In some aspects, the MSCs may be further differentiated to pericytes. In an exemplary method, MSCs are seeded (e.g., at a cell density of 1-20 k/cm^2, particularly 10 k/cm^2 on Tissue Culture Plastic (TCP) 6-well plates) in ScienCell Pericyte Medium (Catalog: 1201) and placed in normoxic incubator conditions. The culture may be fed ScienCell Pericyte Medium every other day until confluent. Confluent cultures may be harvested, such as by using TrypLE. Staining may be performed on harvested cells to detect neural-glial antigen 2/chondroitin sulfate proteoglycan (NG2) and PDGFR-beta (CD140b). Harvested cells may be replated (e.g., at a cell density of 1-20 k/cm^2, particularly 10 k/cm^2 on TCP 6-well plates) in ScienCell Pericyte Medium. The culture may be fed ScienCell Pericyte Medium every other day until confluent, and harvest and stained in the same manner as mentioned. The cells are then replated until there is expansion and retention of purity in cultures. Additionally, the cells may be stained positive for the presence of CD146, CD49a, CD166, CD54, CD73, CD105, CD13, CD56, CD49d, and/or CD44.

G. Differentiation Media

[00168] Cells can be cultured with the nutrients necessary to support the growth of each specific population of cells. Generally, the cells are cultured in growth media including a carbon source, a nitrogen source and a buffer to maintain pH. The medium can also contain fatty acids or lipids, amino acids (such as non-essential amino acids), vitamin(s), growth factors, cytokines, antioxidant substances, pyruvic acid, buffering agents, pH indicators, and inorganic salts. An exemplary growth medium contains a minimal essential media, such as

Dulbecco's Modified Eagle's medium (DMEM) or ESSENTIAL 8™ (E8™) medium, supplemented with various nutrients, such as non-essential amino acids and vitamins, to enhance stem cell growth. Examples of minimal essential media include, but are not limited to, Minimal Essential Medium Eagle (MEM) Alpha medium, Dulbecco's modified Eagle medium (DMEM), RPMI-1640 medium, 199 medium, and F12 medium. Additionally, the minimal essential media may be supplemented with additives such as horse, calf or fetal bovine serum. Alternatively, the medium can be serum free. In other cases, the growth media may contain "knockout serum replacement," referred to herein as a serum-free formulation optimized to grow and maintain undifferentiated cells, such as stem cell, in culture. KNOCKOUT™ serum replacement is disclosed, for example, in U.S. Patent Application No. 2002/0076747, which is incorporated herein by reference. Preferably, the PSCs are cultured in a fully-defined and feeder-free media.

[00169] In some embodiments, the medium may contain or may not contain any alternatives to serum. The alternatives to serum can include materials which appropriately contain albumin (such as lipid-rich albumin, albumin substitutes such as recombinant albumin, plant starch, dextrans and protein hydrolysates), transferrin (or other iron transporters), fatty acids, insulin, collagen precursors, trace elements, 2-mercaptoethanol, 3'-thioglycerol, or equivalents thereto. The alternatives to serum can be prepared by the method disclosed in International Publication No. WO 98/30679, for example. Alternatively, any commercially available materials can be used for more convenience. The commercially available materials include KNOCKOUT™ Serum Replacement (KSR), Chemically-defined Lipid concentrated (Gibco), and GLUTAMAX™ (Gibco).

[00170] Other culturing conditions can be appropriately defined. For example, the culturing temperature can be about 30 to 40°C, for example, at least or about 31, 32, 33, 34, 35, 36, 37, 38, 39°C but particularly not limited to them. In one embodiment, the cells are cultured at 37°C. The CO_2 concentration can be about 1 to 10%, for example, about 2 to 5%, or any range derivable therein. The oxygen tension can be at least, up to, or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20%, or any range derivable therein.

H. Multicellular Cell Cultures

[00171] In certain embodiments, the present disclosure provides multicellular culture models for the study of neuroinflammation, such as to identify novel targets, biomarkers, and therapeutic agents for the diagnosis, prognosis, and treatment of neurodegenerative diseases. In one embodiment, there is provided a triculture of microglia, astrocytes and neurons.

[00172] The neurons may be excitatory and/or inhibitory neurons, either increasing or decreasing activity in the target neuron, respectively. Cortical (GABAergic / inhibitory) neurons, dopaminergic neurons, cholinergic neurons, serotoninergic neurons, glutamatergic neurons, neurons that express strychnine-sensitive glycine receptors, acetylcholine neurons, epinephrine or norepinephrine neurons, or histamine responsive neurons may be used.

[00173] Glutamatergic neurons produce glutamate, which is one of the most common excitatory neurotransmitters in the central nervous system (CNS). It plays a role in fundamental processes, such as learning, cognition, and memory, and dysregulation of glutamatergic transmission can result in several neurological conditions.

[00174] GABAergic neurons produce gamma-Aminobutyric acid (GABA), which is the main inhibitory neurotransmitter in the mammalian central nervous system (CNS). GABA is primarily synthesized from glutamate, catalyzed by glutamate decarboxylase (GAD), and is present at 30 - 40% of synapses. GABA induces either Cl- influx or K+ efflux, resulting in hyperpolarized neurons and reduced action potential. Dysfunction of GABA neurotransmission can result in several disorders, including schizophrenia and epilepsy.

[00175] Dopaminergic neurons of the midbrain are the main source of dopamine (DA) in the mammalian central nervous system. Their loss is associated with one of the most prominent human neurological disorders, Parkinson's disease (PD). In some embodiments, neuronal types typically found in the hippocampus, amygdala, periphery (peripheral neurons), motor neurons, or cortical neurons (*e.g.*, glutamatergic or excitatory neurons) may be used in various embodiments. For example, in some embodiments, cholinergic neurons may be generated as described, *e.g.*, in US 8,513,017 or US 8,796,022. In some embodiments, motor neurons may be generated as described, *e.g.*, in US 8,735,149. In some embodiments, dopaminergic neurons may be generated as described in WO2013067362; WO2013163228; WO2012080248; or WO2011130675.

[00176] The glutamatergic and GABAergic neurons may be positive for SCL1, BCL11B, Calb2, CD24, CDH1, CUX1 Cux2, DCX, DLG4, Dlx, Dlx2, Emx1, Emx2, eomes, ETV1, FOXG1, FOXP2, Fut4, GABRA2, GAD1, GAD2, GAPDH, GFAP, GRIN2B, HoxB4, HTR2C, ISL1, ITGB1, LHX2, Neurog1, NKX2-1, Nos1, NPY, NR4A2, PAX6, POU3F2, PVALB, RELN , SATB2 SLC17A6, SLC17A7, SLC17A8, SLC32A1, SOX1, Sox10, SST, SYN1, and Tbr1. The GABAergic neurons may be characterized by expression of SL3A1, GAD2, and DlX2. The glutamatergic neurons may be characterize by expression of Emx2, SLC17A6, and Tbr1.

[00177] In some embodiments, the following methods may be used to generate GABAergic, glutamatergic, dopaminergic, or cholinergic neurons from pluripotent stem cells such as embryonic stem cells or iPS cells. For example, in some embodiments, the methods of U.S. Patent Application 2012/0276063 may be used to generate neurons from pluripotent stem cells. For example, in some embodiments, bFGF and TGFβ may be excluded from a media (*e.g.*, excluded from a defined media such as TeSR or Essential8 media) that is used to culture pluripotent cells such as iPS cells prior to the start of aggregate formation (while cells were still in adherent culture), then this may be used to promote neuronal differentiation of the pluripotent cells. In some embodiments, when iPS cells are “primed” in the absence of TeSR growth factors, *i.e.*, cultured in any medium that does not have basic fibroblast growth factor (bFGF) and transforming growth factor β (TGFβ), for several days prior to aggregate formation, the cells can develop into the neural lineage with purity, rapidity and consistency. Other methods for making neurons include Zhang *et al.* (2013), US 7,820,439, PCT Publn. No. WO 2011/091048, US 8,153,428, US 8,252,586, and US 8,426,200.

[00178] Cultures of neuronal cell types that are derived from pluripotent cells, including iPS cells, are also commercially available and may be purchased. For example, iCell® Neurons, iCell® DopaNeurons, and iCell® Astrocytes are derived from human iPS cells and may be purchased from Cellular Dynamics International (Madison, Wisconsin). iCell® Neurons are human induced pluripotent stem cell (iPSC)-derived neurons that exhibit biochemical, electrophysiological, and pathophysiological properties characteristic of native human neurons. Due to their high purity, functional relevance, and ease of use, iCell® Neurons represent a very useful *in vitro* test system for neurobiology interrogations in basic research and many areas of drug development.

[00179] In some embodiments, a defined media (*i.e.*, a media that does not contain tissue, feeder cells, or cell-conditioned media) may be used to produce neurons or astrocytes from pluripotent cells such as iPS cells.

[00180] The medium used to produce neurons or astrocytes from iPS cells may also be essentially free of serum and/or serum-derived growth factors. In a further embodiment, the medium may have or be essentially free of externally added TGFβ superfamily signaling modulators, including positive modulators or inhibitors of BMP signaling and/or Activin/Nodal/TGFβ/GDF signaling. For example, a BMP signaling inhibitor may be dorsomorphin and an Activin/Nodal/TGFβ/GDF signaling inhibitor may be SB431542. In a still further embodiment, the medium may have or be essentially free of other externally added FGF signaling modulators, particularly FGF inhibitors.

[00181] In particular embodiments, mature neural cells, including the cultured cells, may be identified as mature neural cells by the expression of one or more of Dcx, MAP-2, Synapsin 1, TuJ1, NSE, Map2a, Gap43, NF, CD24, CDH2/CD325, synaptophysin, and CD56/NCAM. Such a culture of cells can be produced by the methods described herein or by other methods including those later developed.

[00182] Neural cells can be characterized according to a number of phenotypic criteria. The criteria include but are not limited to microscopic observation of morphological features, detection or quantification of expressed cell markers, enzymatic activity, neurotransmitters and their receptors, and electrophysiological function.

[00183] Certain cells that may be used various embodiments have morphological features characteristic of neuronal cells. These features are recognized by those of skill in the art. For example, neurons include small cell bodies, and multiple processes reminiscent of axons and dendrites.

[00184] Neural cells can also be characterized according to whether they express phenotypic markers characteristic of particular kinds of neural cells including but not limited to dopaminergic neurons (markers include TH, AaDC, Dat, Otx-2, FoxA2, LMX1A and VMAT2), cholinergic neurons (markers include NGF, ChAT), GABAergic neurons (markers include GAD67 and vGAT), glutamatergic neurons (markers include vGLUT1), serotonergic neurons, motor neurons (markers include HB9, SMN, ChAT, NKX6), sensory neurons (markers include POU4F1 and peripherin), astrocytes (markers include GFAP and Tapa1), and oligodendrocytes (markers include O1, O4, CNPase, and MBP). The neural cells may express 1, 2, 3, 4, 5, or more markers of a particular kind of neural cell type.

[00185] Astrocytes are a sub-type of glial cells in the central nervous system. They are also known as astrocytic glial cells. Generally star-shaped, their many processes typically envelope synapses made by neurons *in vivo*. Astrocytes are classically identified using histological analysis; many of these cells express the intermediate filament glial fibrillary acidic protein (GFAP). Three forms of astrocytes exist in the CNS, fibrous, protoplasmic, and radial. The fibrous glia are usually located within white matter, have relatively few organelles, and exhibit long unbranched cellular processes. This type often has "vascular feet" that physically connect the cells to the outside of capillary walls when they are in close proximity to them. The protoplasmic glia are found in grey matter tissue, possess a larger quantity of organelles, and exhibit short and highly branched tertiary processes. The radial glia are disposed in a plane perpendicular to the axis of ventricles. Radial glia are predominantly present during development and can play a role in neuron migration *in vivo*. Mueller cells of retina and Bergmann glia cells of cerebellar cortex represent an exception, and are still present during adulthood. A variety of methods may be used to generate astrocytes from pluripotent stem cells such as embryonic stem cells or iPS cells. These methods include, *e.g.*, U.S. Patent Application 2012/0276063, which are incorporated by reference herein in their entirety without disclaimer.

[00186] In some aspects, the cell culture may further comprise exogenous Aβ oligomers, such as synthetic oligomers.

[00187] The cell culture may be a 3D cell culture. The 3D cell culture may be a brain organoid. The term “organoid” as used in the context of this application refers to a three-dimensional cellular structure that mimics the organization and function of organs. Organoids consist of tissue- specific cell types that self-organize through cell sorting and spatially restricted lineage commitment. Organoids may be derived from stem cells, such as embryonic stem cells or induced pluripotent stem cells. The term “brain organoid” refers to an organoid that has anatomical features that resemble that of a brain. It will generally be understood that a brain organoid is comprised of various cell types of the brain. These cell types may have different developmental potential, with some cell types being less differentiated than others. In some aspects, the brain organoid may comprise structures and cell types of retina, cortex, midbrain, hindbrain, brain stem, and/or spinal cord.

[00188] In another embodiment, there is provided blood brain barrier model comprising BMECs, astrocytes, and pericytes. The model may be in a sandwich format comprising BMECs on the apical (i.e., blood) side, a layer of ECM proteins (e.g., collagen IV and fibronectin), and astrocytes and pericytes on the basolateral (i.e., brain) side. The sandwich format may further comprise a permeable membrane insert between the apical and basolateral sides. The ECM proteins may be on the apical side of the membrane. In some aspects, the membrane may be coated with collagen, laminin, proteoglycan, vitronectin, fibronectin, poly-D-lysine and/or polysaccharide. The basolateral side of the membrane may further comprise a layer of gelatin. Alternatively, the basolateral side may be coated with polyalkylene oxides, poloxamines, celluloses, hydroxyalkylated celluloses, polypeptides, polysaccharides, carbohydrates, proteins, copolymers thereof, or combinations thereof, and more particularly are comprised of or derived from poly(ethylene glycol), poly(ethylene oxide), poly(vinyl alcohol), poly(vinylpyrrolidone), poly(ethyloxazoline), poly(ethylene oxide)-co-polypropylene oxide) block copolymers, carboxymethyl cellulose, hydroxyethyl cellulose, methylhydroxypropyl cellulose, polysucrose, hyaluronic acid, dextran, heparan sulfate, chondroitin sulfate, heparin, alginate, collagen, albumin, ovalbumin, copolymers thereof, or a combination thereof.

[00189] In some embodiments, the model may be provided in a microfluidic device. Various microfluidic device configurations useful for the support of cells, including in the form of in vitro blood vessel models, are known in the art. See, e.g., US 2011/0053207 and US 2014/0038279, which are incorporated by reference herein. In general, a microfluidic device comprising the blood brain barrier model as taught herein may comprise a chamber so dimensioned to accept the blood brain barrier model therein such that the endothelial cell layer and neuronal cell layer define a boundary between a first chamber or opening in fluid contact with the endothelial cell layer of the model, and a second chamber or opening in fluid contact with the neuronal cell layer of the model. The fluid may be a liquid such as a media or a buffer. The device may further comprise a fluid inlet and fluid outlet for each chamber, fluid reservoirs (e.g., media reservoirs) connected therewith, etc.

[00190] In some embodiments, the cells used in the cell cultures are generated from iPS cells that were generated from cells obtained from a healthy donor. In other embodiments, the donor has a disease. For example, in some embodiments the donor has a disease such as a neurological or neurodegenerative disease such as, *e.g.*, epilepsy, autism, attention deficit-hyperactivity disorder (ADHD), amyotrophic lateral sclerosis (ALS), Charcot-Marie-Tooth (CMT), Huntington's disease, familial epilepsy, schizophrenia, familial

Alzheimer's disease, Friedreich ataxia, spinocerebellar ataxia, spinal muscular atrophy, hereditary spastic paraparesis, leukodystrophies, phenylketonuria, Tay-Sachs disease, Wilson disease, an addiction disorder, depression, or a mood disorder. The disease may be a genetic disease or an increased genetic susceptibility to a particular neurological disease.

[00191] Further provided herein are assays for studying neuroinflammation using the present cell culture models. The outcome of the triculture system or BBB model can be survival, synaptic pruning, microglia function by Aβ aggregation, p-tau formation, MEA function of neurons, analytes released in the media to trigger the neural inflammation cascade, and cross talk between all three cell types.

[00192] The cells are generally seeded in an appropriate culture vessel, such as a tissue culture plate, such as a flask, 6-well, 24-well, or 96-well plate. A culture vessel used for culturing the cell(s) can include, but is particularly not limited to: flask, flask for tissue culture, dish, petri dish, dish for tissue culture, multi dish, micro plate, micro-well plate, multi plate, multi-well plate, micro slide, chamber slide, tube, tray, CELLSTACK® Chambers, culture bag, and roller bottle, as long as it is capable of culturing the stem cells therein. The cells may be cultured in a volume of at least or about 0.2, 0.5, 1, 2, 5, 10, 20, 30, 40, 50 ml, 100 ml, 150 ml, 200 ml, 250 ml, 300 ml, 350 ml, 400 ml, 450 ml, 500 ml, 550 ml, 600 ml, 800 ml, 1000 ml, 1500 ml, or any range derivable therein, depending on the needs of the culture. In a certain embodiment, the culture vessel may be a bioreactor, which may refer to any device or system *ex vivo* that supports a biologically active environment such that cells can be propagated. The bioreactor may have a volume of at least or about 2, 4, 5, 6, 8, 10, 15, 20, 25, 50, 75, 100, 150, 200, 500 liters, 1, 2, 4, 6, 8, 10, 15 cubic meters, or any range derivable therein.

I. Cryopreservation

[00193] The cells produced by the methods disclosed herein can be cryopreserved, see for example, PCT Publication No. 2012/149484 A2, which is incorporated by reference herein, at any stage of the process, such as Stage I, Stage II, or Stage III. The cells can be cryopreserved with or without a substrate. In several embodiments, the storage temperature ranges from about -50°C to about -60°C, about -60°C to about -70°C, about -70°C to about -80°C, about -80°C to about -90°C, about -90°C to about - 100°C and overlapping ranges thereof. In some embodiments, lower temperatures are used for the storage (*e.g.*, maintenance) of the cryopreserved cells. In several embodiments, liquid nitrogen (or other similar liquid coolant) is used to store the cells. In further embodiments, the cells are stored for greater than about 6 hours. In additional embodiments, the cells are stored about 72 hours. In several embodiments, the cells are stored 48 hours to about one week. In yet other embodiments, the cells are stored for about 1, 2, 3, 4, 5, 6, 7, or 8 weeks. In further embodiments, the cells are stored for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 months. The cells can also be stored for longer times. The cells can be cryopreserved separately or on a substrate, such as any of the substrates disclosed herein.

[00194] In some embodiments, additional cryoprotectants can be used. For example, the cells can be cryopreserved in a cryopreservation solution comprising one or more cryoprotectants, such as DM80, serum albumin, such as human or bovine serum albumin. In certain embodiments, the solution comprises about 1 %, about 1.5%, about 2%, about 2.5%, about 3%, about 4%, about 5%, about 6%, about 7%·, about 8%, about 9%, or about 10% DMSO. In other embodiments, the solution comprises about 1% to about 3%, about 2% to about 4%, about 3% to about 5%, about 4% to about 6%, about 5% to about 7%, about 6% to about 8%, about 7% to about 9%, or about 8%· to about 10% dimethylsulfoxide (DMSO) or albumin. In a specific embodiment, the solution comprises 2.5% DMSO. In another specific embodiment, the solution comprises 10% DMSO.

[00195] Cells may be cooled, for example, at about 1° C/minute during cryopreservation. In some embodiments, the cryopreservation temperature is about -80° C to about -180° C, or about -125° C to about -140° C. In some embodiments, the cells are cooled to 4 °C prior to cooling at about 1 °C/minute. Cryopreserved cells can be transferred to vapor phase of liquid nitrogen prior to thawing for use. In some embodiments, for example, once the cells have reached about -80° C, they are transferred to a liquid nitrogen storage area. Cryopreservation can also be done using a controlled-rate freezer. Cryopreserved cells may be thawed, *e.g.*, at a temperature of about 25° C to about 40° C, and typically at a temperature of about 37° C.

III. Methods of Use

[00196] The present disclosure provides a method by which large numbers of cells of multiple lineages can be produced as well as compositions comprising cell of the CNS. These cell populations can be used for a number of important research, development, and commercial purposes. These include, but are not limited to, transplantation or implantation of the cells *in vivo*; screening anti-virals, cytotoxic compounds, carcinogens, mutagens, growth/regulatory factors, pharmaceutical compounds, *etc.*, *in vitro*; elucidating the mechanism of liver diseases and infections; studying the mechanism by which drugs and/or growth factors operate; diagnosing and monitoring cancer in a patient; gene therapy; and the production of biologically active products, to name but a few.

[00197] Multicellular cultures provided herein may be used, *e.g.*, in testing the effect of molecules on neural differentiation or survival, or in toxicity testing or in testing molecules for their effects on neural or neuronal functions. This can include screens to identify compounds that affect neuron activity, plasticity (*e.g.*, long-term potentiation), or function. The cell cultures may be used in the discovery, development and testing of new drugs and compounds that interact with and affect the biology of neural stem cells, neural progenitors or differentiated neural or neuronal cell types. The neural cells can also have great utility in studies designed to identify the cellular and molecular basis of neural development and dysfunction including but not limited to axon guidance, neurodegenerative diseases, neuronal plasticity and learning and memory. Such neurobiology studies may be used to identify novel molecular components of these processes and provide novel uses for existing drugs and compounds, as well as identify new drug targets or drug candidates.

[00198] In some embodiments, one or more specific compounds may be tested to determine if the compound has effects that may be beneficial for the treatment of a disease. Based on the effects of the compound on the functional activity, one may then be able to determine if the compound may be useful for the treatment of a disease. In some embodiments, the cells are derived from iPS cells from a subject that has a disease (*e.g.*, a genetic disease or a disease with a genetic component or risk factor) such as a neuorological or neurodegenerative disease (*e.g.*, autism, epilepsy, ADHD, schizophrenia, bipolar disorder, *etc.*). In some embodiments, the cells may be cultured in the presence of a first compound or toxin so that the neural culture will display properties similar to a disease state; in these embodiments, a second compound may be provided to the cell cultures to see if the second compound can alleviate or reduce the effect of the first compound or toxin. In other embodiments, the cells cultures may be used to determine if a compound produces toxicity or adverse effects on the cell culture.

[00199] For example, one or more candidate agents may be added at varying concentrations to the culture medium. An agent that promotes the expression of a polypeptide of interest expressed in the cell is considered useful; such an agent may be used, for example, as a therapeutic to prevent, delay, ameliorate, stabilize, or treat an injury, disease or disorder characterized by a defect in neurodevelopment or neurological function. Once identified, agents may be used to treat or prevent a neurological condition. In another embodiment, the activity or function of a cell of the organoid is compared in the presence and the absence of a candidate compound. Compounds that desirably alter the activity or function of the cell are selected as useful in the present methods.

[00200] Agents useful in the present methods may be identified from large libraries of natural product or synthetic (or semi-synthetic) extracts or chemical libraries or from polypeptide or nucleic acid libraries, according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the present methods. Agents used in screens may include known those known as therapeutics for the treatment of neurological conditions. Alternatively, virtually any number of unknown chemical extracts or compounds can be screened using the methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as the modification of existing polypeptides.

[00201] The assays to determine functional activity of the cells may comprise survival assays, microglia phagocytosis assays, calcium assays, MEA assays, synaptic pruning by microscopy assays, signal transduction chasing phosphorylated intermediates of various pathways, analysis of analytes released in the media in mono-, bi- or tri-culture with normal and disease specific cell types. For example, for disease modeling applications where isogenically engineered or patient-specific cells are compared to AHN controls, the treatment or exposure to neurogenerative proteins like amyloid beta, myelin, synaptosomes or Tau would result in decreased calcium signaling and electrical activity as well as increased neuroinflammatory cytokines. In some aspects, measuring functional activity comprises measuring dendrite area (e.g., MAP2), synapse count (e.g., Synapsin 1/2), cell count (e.g., CUX2), or axon area (e.g., beta III tubulin). For example, an increase (e.g., more than 30%, 40%, 50%, 60%, 70%, 80%, or 90%) in any of these functional activity measurements may indicate a candidate agent.

[00202] In some aspects, early pathogenic changes may be quantified by observing alterations or predominantly downregulation in gene expression profiles associated with the onset of neurodegeneration. In certain aspects, upregulation of immune related genes associated with the release of neuroinflammatory cytokines may be measured as associated with neurodegeneration. In some aspects, the methods may comprise detecting the level of Gas6-Axl, Siglec-11, ligand gated ion channels, voltage gated ion channels, GPCRs, catalytic receptors, enzymes, nuclear receptors, COMT, NRXN2 and/or SST in microglia. In some aspects, the GPCRs comprise ADGRD1, ADGRE3, ADGRE5, ADGRG1, ADGRG3, ADORA2B, ADRB1, ADRB2, C5AR1, C5AR2, CCR2, CXCR2, CXCR4, EDNRA, FPR3, FZD1, GPBAR1, GPR157, LTB4R, LTB4R2, P2RX1, P2RY1, P2RY12, PTGER4 and/or SUCNR1. In specific aspects, the GPCRs comprise AVPR2, CNR2, GPR18, GPR84 and/or LPAR6. In certain aspects, the ligand-gated ion channels comprise P2XRX1, P2RY12, and/or P2RY10.

[00203] The assays may be performed in a high-throughput manner. For example, the cell cultures can be positioned or placed on a culture dish, flask, roller bottle or plate (e.g., a single multi-well dish or dish such as 8, 16, 32, 64, 96, 384 and 1536 multi-well plate or dish), optionally at defined locations, for identification of potentially therapeutic molecules. Libraries that can be screened include, for example, small molecule libraries, siRNA libraries, and adenoviral transfection vector libraries. The screening platform may be automated, such as robotic automation. The culturing platform may comprise an automated cell washer and high content imager.

[00204] In some aspects, the present assay may quantify the response of the present cell cultures to different neuroinflammatory stimuli mimicking sterile bacterial infection (lipopolysaccharide (LPS) exposure), mechanical injury (scratch), and seizure activity (glutamate-induced excitotoxicity). The secreted cytokine profile of control and LPS-exposed cultures may be measured

[00205] The blood brain barrier models as described herein may be used for compound or treatment screening or testing (e.g., for efficacy, toxicity, or other metabolic or physiological activity) for pharmacodynamic or pharmacokinetic testing of the passage of agents through the blood brain barrier, etc. Such testing may be carried out by providing an *in vitro* blood brain barrier model as described herein under conditions which maintain constituent cells of that product alive (e.g., in a culture media with oxygenation); applying a compound to be tested (e.g., a drug candidate) to the cells (e.g., by administration to the endothelial layer); and then detecting a penetration of the compound through the endothelial layer and/or other physiological response (e.g., damage, scar tissue formation, infection, cell proliferation, bum, cell death, marker release such as histamine release, cytokine release, changes in gene expression, etc.), which may indicate whether said compound can penetrate the blood brain barrier and/or has therapeutic efficacy, toxicity, or other metabolic or physiological activity in the brain if systemically delivered (e.g., intravascularly) to a mammalian subject. A control sample of the blood brain barrier may be maintained under like conditions, to which a control compound (e.g., physiological saline, compound vehicle or carrier) may be applied, so that a comparative result is achieved, or damage can be determined based on comparison to historic data, or comparison to data obtained by application of dilute levels of the test compound, etc.

[00206] Methods of determining whether a test compound has immunological activity may include testing for immunoglobulin generation, chemokine generation and cytokine generation by the microglia or astrocytes of the blood brain barrier model or by assessing migration of innate immune cells such as the neutrophils, and macrophages into the neuronal layer.

[00207] Methods of crossing the blood brain barrier (e.g., the human blood brain barrier) that may be tested with the models taught herein include, but are not limited to, assessing permeability of different paracellular tight junctions, passive diffusion through the cell layers, receptor-mediated transcytosis, and/or cell efflux inhibition.

[00208] In some embodiments, the model may be used in personalized testing of a subject (e.g., for efficacy, toxicity, or other metabolic or physiological activity) for pharmacodynamic or pharmacokinetic testing of the passage of agents through the blood brain barrier, etc., with at least some of the cells of the model being from the subject. For example, fibroblast cells of the subject may be directed to induced pluripotent stem cells (e.g., induced pluripotent neural stem cells), which cells thereafter are directed to one or more cell types for the model, e.g., neuronal cells, oligodendrocytes, endothelial cells, astrocytes, or microglia.

[00209] Further provided herein methods of treating a neurodegenerative disease by administering a TREM2 agonist. The TREM2 agonist may be an antibody that triggers TREM2 function or an activator of downstream signaling pathways trigged by TREM2, such as spleen tyrosine kinase (pSyk), PI3K, TYRO protein tyrosine kinase binding protein (TYROBP), DNAX-activating protein of 12 kDa (DAP12), AKT kinase, or PLCγ pathways.

[00210] The term “neurodegenerative disease or disorder” and “neurological disorders” encompass a disease or disorder in which the peripheral nervous system or the central nervous system is principally involved. The compounds, compositions, and methods provided herein may be used in the treatment of neurological or neurodegenerative diseases and disorders. As used herein, the terms “neurodegenerative disease”, “neurodegenerative disorder”, “neurological disease”, and “neurological disorder” are used interchangeably.

[00211] Examples of neurological disorders or diseases include, but are not limited to chronic neurological diseases such as diabetic peripheral neuropathy (including third nerve palsy, mononeuropathy, mononeuropathy multiplex, diabetic amyotrophy, autonomic neuropathy and thoracoabdominal neuropathy), Alzheimer's disease, age-related memory loss, senility, age-related dementia, Pick's disease, diffuse Lewy body disease, progressive supranuclear palsy (Steel-Richardson syndrome), multisystem degeneration (Shy-Drager syndrome), motor neuron diseases including amyotrophic lateral sclerosis (“ALS”), degenerative ataxias, cortical basal degeneration, ALS-Parkinson's-Dementia complex of Guam, subacute sclerosing panencephalitis, Huntington's disease, Parkinson' s disease, multiple sclerosis (“ MS”), synucleinopathies, primary progressive aphasia, striatonigral degeneration, Machado-Joseph disease/spinocerebellar ataxia type 3 and olivopontocerebellar degenerations, Gilles De La Tourette's disease, bulbar and pseudobulbar palsy, spinal and spinobulbar muscular atrophy (Kennedy's disease), primary lateral sclerosis, familial spastic paraplegia, Wernicke-Korsakoff's related dementia (alcohol induced dementia), Werdnig-Hoffmann disease, Kugelberg-Welander disease, Tay-Sach's disease, Sandhoff disease, familial spastic disease, Wohifart-Kugelberg-Welander disease, spastic paraparesis, progressive multifocal leukoencephalopathy, and prion diseases (including Creutzfeldt-Jakob, Gerstmann-Straussler-Scheinker disease, Kuru and fatal familial insomnia). Other conditions also included within the methods of the present disclosure include age-related dementia and other dementias, and conditions with memory loss including vascular dementia, diffuse white matter disease (Binswanger's disease), dementia of endocrine or metabolic origin, dementia of head trauma and diffuse brain damage, dementia pugilistica, and frontal lobe dementia. Also other neurodegenerative disorders resulting from cerebral ischemia or infarction including embolic occlusion and thrombotic occlusion as well as intracranial hemorrhage of any type (including, but not limited to, epidural, subdural, subarachnoid, and intracerebral), and intracranial and intravertebral lesions (including, but not limited to, contusion, penetration, shear, compression, and laceration). Thus, the term also encompasses acute neurodegenerative disorders such as those involving stroke, traumatic brain injury, schizophrenia, peripheral nerve damage, hypoglycemia, spinal cord injury, epilepsy, and anoxia and hypoxia.

A. Pharmaceutical Compositions

[00212] Also provided herein are pharmaceutical compositions and formulations comprising the present cells and a pharmaceutically acceptable carrier.

[00213] Cell compositions for administration to a subject in accordance with the present invention thus may be formulated in any conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

[00214] Pharmaceutical compositions and formulations as described herein can be prepared by mixing the active ingredients (such as cells) having the desired degree of purity with one or more optional pharmaceutically acceptable carriers (Remington's Pharmaceutical Sciences 22nd edition, 2012), in the form of lyophilized formulations or aqueous solutions. Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG). Exemplary pharmaceutically acceptable carriers herein further include insterstitial drug dispersion agents such as soluble neutral-active hyaluronidase glycoproteins (sHASEGP), for example, human soluble PH-20 hyaluronidase glycoproteins, such as rHuPH20 (HYLENEX®, Baxter International, Inc.). Certain exemplary sHASEGPs and methods of use, including rHuPH20, are described in U.S. Patent Publication Nos. 2005/0260186 and 2006/0104968. In one aspect, a sHASEGP is combined with one or more additional glycosaminoglycanases such as chondroitinases.

B. Distribution for Commercial, Therapeutic, and Research Purposes

[00215] In some embodiments, a reagent system is provided that includes cells that exists at any time during manufacture, distribution or use. The kits may comprise any combination of the cells described in the present disclosure in combination with undifferentiated pluripotent stem cells or other differentiated cell types, often sharing the same genome. Each cell type may be packaged together, or in separate containers in the same facility, or at different locations, at the same or different times, under control of the same entity or different entities sharing a business relationship. Pharmaceutical compositions may optionally be packaged in a suitable container with written instructions for a desired purpose, such as the mechanistic toxicology.

[00216] In some embodiments, a kit that can include, for example, one or more media and components for the production of cells is provided. The reagent system may be packaged either in aqueous media or in lyophilized form, where appropriate. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there is more than one component in the kit, the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial. The components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means. The kits of the present disclosure also will typically include a means for containing the kit component(s) in close confinement for commercial sale. Such containers may include injection or blow molded plastic containers into which the desired vials are retained. The kit can also include instructions for use, such as in printed or electronic format, such as digital format.

IV. Examples

[00217] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1 – Generation of Endothelial Cells

[00218] iPSCs maintained on MATRIGEL™ or Vitronectin in the presence of E8, were adapted to hypoxia for at least 5-10 passages. Cells were split from sub confluent iPSCs and plated at a density of 0.25 million cells/well onto Amine culture dishes in the presence Serum Free Defined (SFD) media supplemented with 5 uM blebbistatin or 1 uM H1152. 24 hrs post plating SFD media supplemented with 50ng/ml of BMP4, VEGF and FGF2 is added to the culture. The cells are fed every 48 hrs throughout the differentiation process. The entire process is performed under hypoxic conditions. The cells are harvested at the end of differentiation and can be cryopreserved or replated on a carboxyl surface at a density of 25k/cm^2 to initiate endothelial differentiation in the presence of VascuLife VEGF Endothelial Media or SFD Endothelial medium (FIG. 2).

[00219] Cryopreserved day 6 HPCs or live cultures are plated at 25k/cm^2 on a carboxyl surface in the presence of VascuLife VEGF Endothelial Medium in the presence of 1μM H1152 and hypoxic conditions. The cells were given a fresh feed of VascuLife 24 hrs post plating and the cultures were fed every 48 hours until they reached confluency. It took 5-6 days for cells to reach confluency. The cells were harvested using Accumax with minimal agitation or pipetting, stained for surface endothelial markers CD31, CD105 and CD144 and replated onto a carboxyl surface at 25k/cm^2 with VascuLife + H1152 and placed in hypoxic incubator conditions. The cells were given a full feed of VascuLife on days 2, 4, 6 post-split. On day 7, the cells were harvested, stained, and replated in the same manner three more times. The histogram depicts the increasing purity of endothelial cells at each replate stage. Pure endothelial cells were generated using successive passage purification without the use of CD31+ MACS. The endothelial cells can be cryopreserved at the end of replate passage 3 (FIG. 3).

Example 2 – Generation of Mesenchymal Stem Cells

[00220] FIG. 5C shows a schematic representation of the 2D HPC differentiation process to generate MSCs. iPSCs maintained on MATRIGEL™ or Vitronectin in the presence of E8, were adapted to hypoxia for at least 5-10 passages. Cells were split from sub confluent iPSCs and plated at a density of 0.25 million cells/well onto Amine culture dishes in the presence Serum Free Defined (SFD) media supplemented with 5 uM blebbistatin or 1 uM H1152. 24 hrs post plating SFD media supplemented with 50ng/ml of BMP4, VEGF and FGF2 was added to the culture. The cells were fed every 48 hrs throughout the differentiation process. The entire process was performed under hypoxic conditions. At the end of day 6 /7 of differentiation the cells were placed in GMP –MSC media. The phenotype of the precursor population was analyzed post-harvest (Figure 5C). The cells were allowed to grow to confluency and harvested at the end of each passage, then replated on an Amine surface at a density of 50K/cm^2 in GMP-MSC Media supplemented with 5 uM blebbistatin or 1 uM H1152 to selectively allow the growth and proliferation of MSCs.

[00221] Cryopreserved Day 6 HPCs from 3D/2D HPC differentiation or live cultures at the end of day 6 differentiation emerging from 2D HPC differentiation were placed in the presence of MSC media in the presence of 1uM H1152 on an Amine charged surface plates. The cells were given a fresh feed of MSC media 24 hrs post plating and the cultures were fed every 48 hours until they reached confluency. It took 5-6 days for cells to reach confluency. The cells were harvested using TrypLE, and stained for surface MSC markers CD73, CD44, CD105, CD49d, and the absence of Endothelial markers CD31 and CD144. The emerging cultures were passaged three times using the process described above under hypoxic conditions and Amine surface. The cultures were transitioned to normoxia and normal tissue culture plates at P4. The purity spec for MSCs was reached at P6 (FIG. 6, 7). Cryopreserved MSCs at P3 were thawed and placed in to lineage specific differentiation matrix as described in FIG. 8A to demonstrate tri-lineage potential to generate Osteocytes, Chondrocytes and Adipocytes (FIG. 8B). The clonal proliferative capacity of cryopreserved MSCs was demonstrated by plating MSCs at a density of 1000 cells/cm^2 in 10cm tissue culture plates. The cells were fed with MSC media for 2 weeks with media changes every alternate day. The emerging colonies were stained with crystal violet and scored (FIG. 8C).

Example 3 – Generation of Pericytes from MSCs

[00222] iCell MSCs and iPSC-derived pericytes were sampled at 50% confluency and analyzed by flow cytometry for known pericyte markers PDGFRβ, NG2, and CD146. Cryopreserved MSCs were thawed and plated at 35,000 cells/cm2 in 6-well plates with no extracellular matrix (ECM) in MSC Maintenance Medium (FIG. 9A). The cells were allowed to reach confluency and the cells were replated at 15,000 cells/cm2 in 6-well plates with no extracellular matrix (ECM) in SFD Pericyte Medium (SPM) (FIG. 9A; FIG. 9B).

[00223] Primary Human Brain Vascular Pericytes (HBVP) (ScienCell # 1200) were thawed and plated on Poly-L-Ornithine coated 6-well plates at 5,000 cells/cm^2 in Pericyte Medium (ScienCell # 1201). These cells were used as a positive control in the differentiation process. ScienCell HBVPs, iCell MSCs, and iPSC-derived pericytes were analyzed by flow cytometry for known pericyte markers PDGFRβ, NG2, and CD146 (FIG. 9C). There was an absence of pericyte markers of iCell MSCs at thaw. HBVP and iPSC-derived pericytes show expression of known pericyte markers PDGFRβ, NG2, and CD146, with iPSC-derived pericytes having greater purity than ScienCell HBVPs (FIG. 9C). iPSC-derived pericytes exhibit similar morphology to ScienCell HBVPs (FIG. 9D).

[00224] Based on their function, pericytes can be classified as phenotypically PC1 (pro-inflammatory) or PC2 (contractile) (Rustenhoven *et al.*, 2017). The signature of both subtypes is described in FIG. 9E. Post-thaw, iPSC-derived pericytes were subtyped via flow cytometry for PC1 and PC2 markers CD274, VCAM1, Calponin, Desmin, DLK1, and αSMA (FIG. 9F). iPSC-derived pericytes reveal the signature of contractile pericytes, subtype PC2.

[00225] In addition to non-specific phagocytic uptake seen in chronic and acute BBB models, pericytes also specifically regulate their neuronal microenvironment by handling clearance of certain macromolecules in both physiologic and pathologic conditions (Winkler *et al.,* 2014). iPSC-derived pericytes were plated at 15,000 cells/cm^2 in a 96-well plate with PDL-coating (Greiner # 655946) in SPM. The cells were allowed to rest for three days post-plating before dead indicator NucGreen Dead 488 (Invitrogen # R37109) and S. aureus pHrodo Red BioParticles (Invitrogen # A10010) were added to the cells. The plate was placed on an IncuCyte live imaging system for over a month, with weekly feeds (including same concentration of live/dead and bioparticle reagents). iPSC-derived pericytes show observable phagocytic activity of S. aureus bioparticles above controls.

Example 4 - Generation of Brain Microvascular Endothelial Cells (BMECs)

[00226] iPSCs maintained on MATRIGEL™ or Vitronectin in the presence of E8 were adapted to hypoxia for at least 5-10 passages for generating Brain Microvascular Endothelial cells. Live or cryopreserved HPCs (e.g., day 6 HPCs derived on Amine surface in the presence of SFD supplemented with BMP4, VEGF and/or FGF2, such as BMP4 and FGF2) were plated onto a ECM containing fibronectin (e.g., 50-200 µg/mL, particularly 100 µg/mL) and Collagen IV (e.g., 100-500 µg/mL, particularly 400 µg/mL) with ECRA Medium (Human Endothelial SFM (Gibco), 1% Platelet-poor plasma-derived bovine serum (Fisher), 20ng/mL bFGF (Promega), 10uM Retinoic Acid). The cells were plated at a density of 50-100 k/cm2, particularly 75 k/cm2. The cultures were fed daily and maintained under hypoxic incubator conditions. The cultures are fed with ECRA Medium every other day until confluent. Confluent cultures are then harvested, such as by using TrypLE. Staining was performed on harvested cells to detect PECAM-1 (CD31) and GLUT-1 to confirm the identity of BMECs (FIG. 10B). Harvested cells are replated, such as on Transwell inserts with ECRA Medium and placed in hypoxic incubator conditions. (FIG. 10A). The culture may be fed ECRA Medium every other day until confluent. Confluent cultures may be tested for the presence of P-gp, CD105, Glu-1 and CD31 expression by flow cytometry (FIG. 10C) and immunocytochemistry (FIG. 10D) and trans-endothelial electrical resistance (TEER) and compared to blank media (FIG. 10E). For the immunohistochemistry cells were washed with 200 µl DPBS 3 times, then incubated with Rabbit anti-P-gp antibody (1:50 in blocking buffer (10%FBS, 0.01% TritonX in DPBS)) at 4°C for overnight. After washing with 200 µl DPBS 3 times, P-gp was stained with secondary antibody (1:1000, Donkey anti-Rabbit IgG Alexa Fluor 488 (Invitrogen)). Nuclei was stained with Hoechst3342 (Thermo Fisher) The image was captured at 200X magnification by ImageXpress (Molecular Devices, LLC).

Example 5 - Generation of Microglia

[00227] iPSCs maintained on MATRIGEL™ or Vitronectin in the presence of E8 were adapted to hypoxia for at least 5-10 passages. 2D HPC Differentiation: Cells were split from sub confluent iPSCs and plated at a density of 0.25-0.5 million cells/well onto Amine culture dishes in the presence Serum Free Defined (SFD) media supplemented with 5 uM blebbistatin or 1uM H1152. 24 hrs post plating SFD media supplemented with 50 ng/ml of BMP4, VEGF and FGF2 was added to the culture. The following day a full media exchange was performed.

[00228] On the fifth day of the differentiation process the cells were placed in media containing 50 ng/ml Flt-3 Ligand, SCF, TP0, IL3 and IL6 with 5U/ml of heparin. The cells were fed every 48 hrs throughout the differentiation process. The entire process was performed under hypoxic conditions. HPCs were quantified by the presence of CD43/CD34 cells.

[00229] 3D HPC Differentiation: Cells were split from sub confluent iPSCs and plated at a density of 0.25-0.5 million cells per ml into a spinner flask in the presence of Serum Free Defined (SFD) media supplemented with 5 uM blebbistatin or 1uM H1152. 24 hours post plating SFD media supplemented with 50 ng/ml of BMP4, VEGF and FGF2 was exchanged. On the fifth day of the differentiation process the cells were placed in media containing 50ng/ml Flt-3 Ligand, SCF, TP0, IL3 and IL6 with 5U/ml of heparin. The cells were fed every 48 hours throughout the differentiation process. The entire process was performed under hypoxic conditions. HPCs were quantified by presence of CD43/CD34. The process outlines and the efficiency is illustrated in (FIG. 11) and the media compositions are listed in (FIG. 12).

[00230] HPCs were placed in microglia differentiation media MDM OR 2X-MDM (FIG. 11). The cultures were fed using every 48 hours. The purity markers for microglial cultures on day 23 of differentiation was quantified before and after cryopreservation (FIG. 13A, FIG. 13B).

[00231] Day 23 live and cryopreserved microglia cultures were assessed for purity. Microglia cultures on day 23 differentiation were harvested and stained for the presence of microglia specific markers. The remaining cells were cryopreserved using a control rate freezer. The cryopreserved cells were thawed and stained for the presence of microglia specific markers. For both sets cell surface expression of CD45, CD33, TREM2, and CD11b (FIG. 14A) as well as intracellular expression of PU.1, IBA, P2RY12, TREM2 and TMEM119 by flow cytometry (FIG. 14B). The results revealed that cryopreserved microglia retained the purity post cryopreservation.

[00232] HPCs were placed in media to initiate microglia differentiation in the presence of MDM and the intermittent feeds were performed with 2X-MDM. The cells were cryopreserved at day 20, day 23 and day 26 of differentiation using manual freezing protocol or a control rate freezer (CRF). The cryopreserved cells were transferred to liquid nitrogen for a week. Cryopreserved microglia were thawed and placed in microglia maturation medium

(MMM). The cultures were fed every 48 hours with fresh microglia maturation media. The cells were harvested on day 3, 5, 7, 10, 12 and 14-days post thaw and the recovery of viable cells with respect to the initial plating number was quantified (FIGS. 15A-15C).

[00233] Cryopreserved HPCs were differentiated to microglia in the presence of MDM. The total viable number of input HPCs and output microglia was quantified. The process efficiency was calculated based on the purity and absolute number of TREM2 positive cells present on day 23 of microglia differentiation divided by the absolute number of input viable HPCs (FIG. 16).

[00234] Cryopreserved microglia at day 20 (FIG. 17A), day 23 (FIG. 17B) or day 26 (FIG. 17C) of differentiation were thawed in microglia maturation medium (MMM) and fed fresh medium every 48 hours. The total viability and absolute cell number was quantified at days 3, 7 and day 10 post thaw. The data revealed a higher post thaw recovery with day 23 microglia over day 26 microglia (FIGS. 17A-17C).

[00235] Next, functional assessment was performed on cryopreserved microglia on day 20, day 23 and day 26 of the differentiation process. The cells were thawed and plated at 15,000 viable cells/well in a 96 well plate in the presence of 200 μl Microglia Maturation medium per well. The cells were treated with diluted 1 μg/well of opsonized or non-opsonized pHrodo Red BioParticles (Thermo Fisher # A10010, 2 mg per vial; stored at -20°C). The plate was placed on the IncuCyte and images of the phagocytosis were taken at various time points up to 5 days post thaw. Cells cryopreserved via control rate freezer method exhibit more robust phagocytosis (due to higher cell viability (FIG. 19)).

[00236] The functional assessment was extended to later time points post thaw. The phagocytic potential was assessed at day 5, day 7 and day 14 post thaw for cryopreserved microglia at day 20, 23 and 26 day of differentiation using the manual or control rate freezer assessed via live imaging on the IncuCyte system. Cryopreserved microglia were thawed and plated in MMM for three days. The viable cell counts at the end of three days were determined as described in Figure 18B. 15,000 viable cells were plated in a 96 well plate in the presence of 200 μl Microglia Maturation medium (MMM) per well with diluted 1 μg/well of opsonized or non-opsonized pHrodo Red BioParticles and the plate was placed on the IncuCyte and images of the phagocytosis were taken at various time points up to 5, 7 and 14 days post thaw.

Manual cryopreservation method revealed decreased / right-shifted rate of phagocytosis across all conditions (due to lower cell viability).

[00237] Phagocytic index is a measure of phagocytic activity determined by counting the number of bacteria ingested per phagocyte during a limited period of incubation of a suspension of bacteria and phagocytes. The ability of cryopreserved microglia to engulf labeled bacterial particles was quantified by the ratio of the number of phagocytosis red object count/ total live cells. This ratio was determined as the phagocytic index (FIG. 21).

[00238] Cryopreserved microglia were thawed and plated at 15,000 viable cells/well in a 96 well plate in the presence of 200 µl Microglia Maturation medium per well. The cells were treated with diluted 1 µg/well of opsonized or non-opsonized pHrodo Red BioParticles (Thermo Fisher # A10010, 2 mg per vial; stored at -20°C). The plate was placed on the IncuCyte and images of the phagocytosis were taken at various time points up to 5 days post thaw. Cells cryopreserved via control rate freezer method exhibit more robust phagocytosis (due to higher cell viability (FIG. 22).

[00239] Next, the differentiation of HPCs to microglia was further developed in the absence of ECM and in a 96-well format amenable to screening application. The differentiation as performed on Ultra-low attachment (ULA), Tissue Culture (TC) and Non-tissue culture (Non-TC) vessels (FIG. 23A). Cryopreserved HPCs were plated at a density of 20,000-35,000 viable cells/cm2 on a 96 well Primaria plate or Ultra-low attachment, tissue culture (TC) or non-tissue culture plates (Non-TC) in the presence of 200ul microglia differentiation medium per well (FIGS. 23B). The cells were fed every 48 hrs with 50 µl media per well of MDM for the next 23 days of differentiation. The cells were harvested with cold PBS on day 23 and the total viable cell number was quantified using an automated cell counter. The cells were stained for surface expression of CD11b, CD45, CD33, TREM2 and intracellular expression of TREM2, IBA, P2RY12 and TMEM119 (FIGS. 24A-24B).

[00240] **Table 1:** Process Efficiency of generating microglia on charged surfaces.

Plate Type	Day 0 Cell Number	Day23 Cell Number	Expansion
Primera	0.684X106	4.0X106	5.8x
ULA	0.684X106	2.06X106	3x
TC	0.684X106	3.22X106	4.7x
Non-TC	0.684X106	3.52X106	5.1x

[00241] Cytokines and Chemokines released by cryopreserved microglia. Day 23 cryopreserved microglia were thawed into MDM medium and plated on to Primaria 96 well plate at 50,000 cells/well. Cells were plated for three days prior to starting stimulations with 100 ng/ml LPS and 50 ng/ml interferon gamma. Stimulations were performed in triplicate for 24 hours. Supernatant was spun down to remove cells and debris and immediately placed at -20°C. The supernatants were analyzed on a multiplex Luminex assay.

Example 6 - Engineering iPSCs to generate variants mimicking neurodegenerative diseases

[00242] TREM2 function was disrupted by introducing indels in exon 2, leading to frameshift and premature translation termination. TAL-nucleases (pair TREM2 below) were designed to bind DNA sequences centered around amino acid 58 within exon 2. The cell line used for engineering was the FCDI iPSC line 01279.107. TAL nuclease mRNA and a co-selection plasmid expressing blasticidin resistance under the control of the SV40 promoter were electroporated into cells using a BioRad Gene Pulser Xcell system with settings of 125V/950uF. The cells were plated and a short blasticidin selection was applied on days 1 and 2 post electroporation. Surviving cells were grown and then single-cell sorted into 96 well plate on day 7 post electroporation. After about two weeks, 81 clones were picked and genotyped by PCR and sequencing.

[00243] Of 81 clones sequenced, 7 showed sequence modification. Three clones contained one allele with an insertion of one base pair, three clones contained one allele with a deletion of one base pair, and one clone was a compound heterozygote with one allele containing a one base pair insertion and one allele containing a four base pair deletion. The seventh clone contained a deletion of 24 base pairs that was not expected to introduce a frameshift. Clones were expanded, cryopreserved, and underwent sequence confirmation and karyotype analysis. After differentiation into microglia two main clones were chosen as example Heterozygous or Homozygous disruptions. The Heterozygous clone 01279.1185 contained an allele with the 1 bp insertion, leading to a frameshift at position 60 of TREM2 and termination after 45 following amino acids. The Homozygous clone 01279.1187 contained an allele with a 1bp frameshifting insertion at position 59 and termination 16 amino acids later, and the second allele having a 4 bp deletion at position 59 leading to a frameshift and termination 46 amino acids later.

[00244] **Table 2:** Target sequences.

TAL	Target Sequence
TREM2-F	GGTGCCGCCAGCTGGGAG (SEQ ID NO:1)
TREM2-R	GCGTGCTGACCACACGCT (SEQ ID NO:2)

[00245] **Table 3**.

Name of Cell Line (Descriptive)	iPSC Number	DNA variant Description	Predicted Protein variant description
TREM2 Heterozygous KO	01279.1185	NM_018965.3:c.[280dupC];[280_281=]	NP_061838.1:p.[(Cys60MetfsTer45)];[(=)]
TREM2 Homozygous KO	01279.1187	NM_018965.3:c.[278_279insG];[278-281delCCCA]	NP_061838.1:p.[(Pro59AlafsTer16)];[(Pro59AlafsTer46)]

Example 7 - Generation of additional isogenically engineered lines mimicking neurodegeneration:

[00246] A Parkinson's Disease model was produced by genetically engineering episomally reprogrammed iPSC 01279 by nuclease-mediated homologous recombination and a donor oligo SJD 14-133. The resulting iPSCs contained SNP rs104893877 where amino acid 53 was changed from alanine to threonine resulting in the A53T variant in the alpha-synuclein gene (SNCA) as well as two silent mutations resulting in the SNCA A53T iPSC line.

[00247] An isogenically engineered model for studying Rett Syndrome was generated by using nuclease-mediated homologous recombination and a donor plasmid p1553. Donor plasmid p1553 inserted a series of stop codons prior to the Methyl CpG Binding domain followed by a PGKp-PuromycinR-SV40pA selection cassette flanked by LoxP sites. The

MECP2 HM line derived from parental line 01279 provided a disease model for Rett Syndrome.

[00248] Generation of HPCs and microglia from isogenic engineered iPSCs: Homozygous and Heterozygous TREM2 KO iPSCs derived from 01279 iPSC along with SNCA A53T and MECP2 HM engineered lines derived from 01279 were maintained in the presence of E8 and MATRIGEL™ acclimatized to hypoxic conditions by passaging them for 10 passages. The cells were karyotyped, and iPSC banks were made to initiate HPC differentiation via the 3D HPC differentiation protocol. Cells were split from sub confluent iPSCs and plated at a density of 0.25-0.5 million cells per ml into a spinner flask in the presence of Serum Free Defined (SFD) media supplemented with 5 µM blebbistatin or 1µM H1152. 24 hrs post plating SFD media supplemented with 50 ng/ml of BMP4, VEGF and FGF2 is exchanged. On the fifth day of the differentiation process the cells are placed in media containing 50 ng/ml Flt-3 Ligand, SCF, TP0, IL3 and IL6 with 5-10 U/ml of heparin. The cells are fed every 48 hrs throughout the 13 day differentiation process. The entire process is performed under hypoxic conditions. HPCs quantified by presence of CD43/CD34. HPCs are cryopreserved post MACs sorting using CD34 beads. Microglia were generated by thawing cryopreserved HPCs and placing the cells in a 23-day differentiation process as described in Example 5.

[00249] Cryopreserved microglia from day 23 wild type and TREM engineered clones were thawed and the presence of TREM-2 expression along with CD45 was quantified by flow cytometry (FIG. 26).

[00250] Cryopreserved day 23 microglia derived from isogenically engineered lines were thawed and stained for the presence of microglia specific markers. The cells were stained to quantify cell surface expression of CD45, CD33, TREM2, and CD11b as well as intracellular expression of PU.1, IBA, P2RY12, TREM2 and TMEM119 proteins by flow cytometry. FIG. 26 summarizes the purity obtained across all four isogenically engineered iPSCs. The results demonstrate the generation of highly pure microglia from isogenically engineered iPSCs with no alteration in the differentiation protocol.

[00251] Levels of soluble TREM2 (sTREM2) protein secreted by microglia post thaw were quantified from conditioned media collected from WT and TREM2 Heterozygous and Homozygous KO mutants using a Simple Step ELISA (AbCam) (FIG. 27A). WT and

TREM2 KO microglia were thawed and plated at the same density in maturation media in a 96 well Primaria plate. The spent media was collected at Day 3 post thaw and day 7 post thaw. The cultures were half fed with fresh maturation media on day 3 and Day 5 post thaw. The data revealed a difference in the levels of soluble TREM2 between WT, Heterozygous TREM2 KO and Homozygous KO microglia. This assay can be used as a functional assay to distinguish WT and TREM2 engineered iPSCs.

[00252] Levels of soluble TREM2 (sTREM2) protein secreted by microglia post thaw were quantified from conditioned media collected from WT and TREM2 Heterozygous and Homozygous KO mutants, MECP2HM and SNCA-A53T using a Simple Step ELISA (AbCam) (FIG. 27A). WT and TREM2 KO microglia were thawed and plated at the same density in maturation media in a 96 well Primaria plate. The spent media was collected at Day 3 post thaw and day 7 post thaw. The cultures were half fed with fresh maturation media on day 3 and Day 5 post thaw. The data revealed a difference in the levels of soluble TREM2 between WT, Heterozygous TREM2 KO and Homozygous KO microglia. This assay can be used as a functional assay to distinguish WT and TREM2 engineered iPSCs. The release soluble TREM2 was impaired in A53T-SNCA microglia while MECP2HM microglia did reveal any alterations in the levels of sTREM released in the media (FIG. 27B).

[00253] Cytokines and Chemokines released by isogenically engineered cryopreserved microglia. Day 23 cryopreserved microglia derived from WT, 1185 HT TREM2 KO, 1187 HO TREM2 KO A53T-SNCA and, MeCP2HM microglia were thawed into MDM medium and plated on to Primaria 96 well plate at 50,000 cells/well. Cells were plated for three days prior to starting stimulations with 100 ng/ml LPS to check M1 mediated response. Stimulations were performed in triplicate for 24 hours. Supernatant was spun down to remove cells and debris and immediately placed at -20°C. The supernatants were analyzed on a multiplex Luminex assay. The results of this multiplex Luminex assay is captured as a heat map in FIG. 27C. Engineered lines secreted a higher level of IL-6 compared to ANH control. TREM2 HZ and TREM2 HO and MeCP2HM microglia released less TNF alpha, but increased levels of IL6 compared to ANH. A53T-SNCA microglia released similar levels of IL-6 and TNF alpha compared to AHN control microglia.

[00254] All engineered lines released the M2 cytokine IL-10 when treated with an M1 stimulus (LPS). MECP2HM microglia released less IL-10 compared to the AHN control microglia (FIG. 27E). AHN and engineered microglia were capable to release CCL2 / MCP-1, CCL20 / MIP-3 alpha, CCL4 / MIP-1 beta, CCL5 / RANTES, CX3CL1 / Fractalkine, CXCL1 / GRO alpha, CXCL10 / IP-10, CXCL2 / GRO beta, IL-8 / CXCL8 in response to LPS stimulation. There were some inherent differences in the level of cytokines releases. TREM2HO revealed the highest levels of CCL4 which is key analyte released during the onset of Alzheimer's Disease (AD). MECP2HM, TREM2HZ and TREM2HO microglia released higher levels of CXCL1/ GRO which implies an attempt to recruit assisting cell types granulocytes to aid in microbial killing and trigger an inflammatory response during phagocytosis, MECPHM microglia revealed a spontaneous secretion of IL-8/CXC18. This analyte is elevated in brain injury and induces the expression of pro-inflammatory proteases and MMP-2 and MMP-9. MECPHM microglia secreted higher levels of IL-6. These results suggest that MECPHM are primed for a pro-inflammatory response. Engineered and AHN microglia released similar levels of PDL-1, CD40, FLT-3 and PDGFAA in the media in response to LPS.

[00255] Cryopreserved microglia were thawed in maturation media and allowed to recover for 48 hrs before screening experiments were performed (FIG. 36). 5,000 microglia from TREM2 WT and TREM2 HOKO were plated per well of a 384 well plate in 40 uL media for 24hrs. In the first set (plate1) the cells were pre-treated with compounds at 1μM final concentration. 24 hours post treating the cells with compounds, pHrodo labelled Amyloid-Beta was added to plate 1 at a final concentration of 1μM and the phagocytosis was captured on an IncuCyteS3 for 96 hrs (FIGS. 37-39). In the second set (plate 2) the cells were plated for 24 hrs followed by treatment with 1ug/ml LPS at a final concentration of 1μg/mL (FIGS. 40-42). 24 hours after exposure to LPS, and 48 hours after initial plating, pHrodo labelled Amyloid - Beta added to plate 2 at a final concentration of 1 μM. Cells were imaged using an IncuCyte once per hour for up to 96 hrs. Phagocytic data was captured as Total Red Object Integrated Intensity X μM2/image. The final volumes for all treatments remained constant. The results of the screen are summarized in FIG. 43.

[00256] To understand the cytokines needed for microglia survival post thaw in the maturation medium a schematic matrix was planned with 32 different media formulations (FIG. 28). WT, 1185 HT TREM2 KO, 1187 HO TREM2 KO microglia were placed at a density of 15,000 viable cell in a 96 well plate in 250 μl of microglia base medium or MMM, or microglia base medium supplemented with a single cytokine (FIG. 29), two cytokines (FIG. 30), three cytokines (FIG. 31), or four cytokines (FIG. 32) in the maturation media. The kinetics of cell survival was captured on the IncuCyte system. NucGreen Dead diluted to 2 drops/mL was added to all well containing cells with various media compositions to capture the number of dead cells with time. The images were captured every 8 hours and the experiment continued for 72 hours without any intermittent feeds. The intensity of the NucGreen Dead quantifies the dead cells in the cultures.

[00257] WT, 1185 HT TREM2 KO, 1187 HO TREM2 KO microglia were placed at a density of 15,000 viable cell in a 96 well plate in 250 µl of microglia base medium (FIG. 33A) or MMM (FIG. 33B), microglia base medium supplemented with IL-34 (26C), microglia base medium supplemented with IL-34 (FIG. 33D), microglia base medium supplemented with MCSF (FIG. 33D), or base medium supplemented with only IL-34 (FIG. 33C) or MSCF or a combination of IL-34 and MCSF (FIG. 33E). The kinetics of cell survival was captured on the IncuCyte system. NucGreen Dead diluted to 2 drops/mL was added to all well containing cells with various media compositions to capture the number of dead cells with time. The images were captured every 8 hours and the experiment continued for 7 days without any intermittent feeds. The intensity of the NucGreen Dead quantifies the dead cells in the cultures

[00258] Functional characterization was assessed on WT, 1185 HT TREM2 KO, 1187 HO TREM2 KO microglia, cryopreserved on day 23 with pHrodo Red labelled bacterial BioParticles and pHrodo Red Amyloid beta. WT, 1185 HT TREM2 KO, 1187 HO TREM2 KO microglia were plated at a density of 15,000-30,000 viable cells/cm^2 in a 96 well plate in 250 µl of MMM (FIGS. 33A-B) or MDM base (AKA microglia base medium) supplemented only MSCF (FIGS. 33C-D) or IL-34 (FIGS. 33E-F) or a combination of IL-34 and MCSF (FIGS. 33G-H) for three days post thaw. The cells were treated with diluted 1 µg/well of opsonized or non-opsonized pHrodo Bioparticles (Thermo Fisher # A10010, 2 mg per vial; stored at -20C) (FIGS. 33A,C,E,G) or pHrodo Amyloid beta (FIGS. 33B,D.F.H). The plate was placed on the IncuCyte and image of the phagocytosis were taken at various time points up to 30 hrs. WT as well as engineered microglia revealed phagocytic function post thaw. The kinetics and the efficiency of phagocytosis varied between the WT, 1185 HT TREM2 KO, 1187 HO TREM2 KO microglia.

[00259] Post thaw purity of day 23 cryopreserved wild type (WT) microglia was determined in the presence of MMM or microglia base medium supplemented with combinations of two critical (IL-34, MSCF) cytokines in the maturation media (FIG. 35). The purity was quantified at day 3, 7 and day 14 post thaw by harvesting the cells and the purity of CD45, CD33, TREM2, CD11b, CX3CR1, P2RY12, TMEM119, IBA was determined at the end of the differentiation process by harvesting the cells and staining the cells for cell surface and intra cellular staining of markers by flow cytometry. Cryopreserved microglia retain viability, purity in maturation media supplemented with MSCF and IL-34. This simplified media will be valuable for co culture applications of cryopreserved microglia with neurons and astrocytes for developing brain organoid models to study the contribution of many SNPs and mutations associated with neurodegeneration.

Example 8 -Generation of disease associated microglia from patient derived iPSCs

[00260] Recent genetic studies have shown that polymorphisms in several microglial-enriched genes are associated with altered risk of developing Alzheimer's disease (AD), Parkinson's disease (PD) and several neural degenerative diseases. Summarizing the list of risk associated SNPs from GWAS studies a panel of end stage microglia were generated from donors exhibiting mutations in TREM2, CD33, and ABCA7, along APOE isoforms. Cryopreserved microglia from patient derived iPSC provide an *in vitro* tool to create a more accurate model to understand complex interactions between human microglia, neurons, astrocytes in a 2D or 3D organoid systems and mimic neurogenerative diseases (McQuade *et al.,* 2019).

[00261] **Generation of HPCs from episomally reprogrammed AHN and disease specific iPSCs**: Episomally reprogrammed iPSC generated from normal as well as disease specific donors were acclimatized to hypoxia for at least 5 -10 passages using E8/ MATRIGEL™ before banking the source material for differentiation towards hematopoietic cells and subsequently to microglia. The genotypes of the panel of iPSCs is described in Table 4. The iPSC derived from all donors were karyotyped, and iPSC banks were made to initiate HPC differentiation via the 3D HPC differentiation protocol was performed as described in example 5. Microglia were generated by thawing cryopreserved HPCs and placing the cells in a 23-day differentiation process as described in Example 5. Cryopreserved day 23 microglia derived from various donors were thawed and stained for the presence of microglia specific markers. The cells were stained for cell surface expression of CD45, CD33, TREM2, and CD11b as well as intracellular expression of PU.1, IBA, P2RY12, TREM2 and TMEM119 proteins by flow cytometry. Table 5 summarizes the purity obtained across all AHN and disease associated microglia (DAM). The results demonstrate the generation of highly pure microglia from a panel of healthy and disease specific donors with no changes in the differentiation protocol.

[00262] Table 4: Generation of cryopreserved Microglia from a panel of Apparently Healthy Normal (ANH) and Disease Associated Microglia (DAM)

			APOE	APOE	APOE	TREM2	CD33
Phenotype	Genotype	Sex	rs429358	rs7412	genotype	Rs7593268	Rs12459419
AHN	N/A	Female	T/T	C/C	3/3	C/C	T/T
AHN	N/A	Female	T/T	C/C	3/3	C/C	T/T
AHN	N/A	Female	T/T	C/C	3/3	C/C	T/T
AHN	TREM2 R47H	Female	T/C	C/T	3/3	C/T	C/C
AHN	N/A	Male	T/T	C/C	3/3	C/C	C/C
AHN	CD33	Female	T/C	C/T	2/4	C/C	T/T
AD	APOE 4/4	Female	C/C	C/C	4/4	C/C	C/C
AD	APOE 4/4	Female	C/C	C/C	4/4	C/C	C/C
AD	APOE 4/4	Female	C/C	C/C	4/4	C/C	C/T
AD	APOE 4/4	Female	C/C	C/C	4/4	C/C	T/T
AHN	ABCA7 G1527A	Male	T/T	C/C	3/3	C/C	C/T
AD	APOE 2/4	Female	T/T	C/C	2/4	C/C	C/T

[00263] Table 5. Overview of purity of microglia apparently healthy normal (ANH) and Alzheimer's Disease (AD) donor samples.

				Percentage Purity Microglia Markers post thaw										
CIRM ID	Phenotype	Genotype	Sex	CD33	CD45	TREM2	P2RY12	TMEM119	CX3CR1	IBA1	CD11c	CD11b	PU.1	S. aureus /Phagocytosis
CW13060BB1	AHN	N/A	Female	93	100	94	81	100	100	82	100	100	99	Yes
CW13038AA1	AHN	N/A	Female	91	100	86	93	99	99	98	99	97	99	Yes
CW13105FF1	AHN	N/A	Female	98	100	98	93	100	99	93	100	99	93	Yes
CW13008DD1	AHN	TREM2 R47H	Female	99	100	98	96	100	99	98	100	100	99	Yes
CW13061EE1	AHN	N/A	Male	99	100	94	92	100	99	99	97	94	94	Yes
CW13042BB1	AHN	CD33	Female	90	100	96	84	96	98	95	100	60	99	Yes
CW13030EE1	AD	APOE E4/E4	Female	98	100	98	98	99	99	98	97	90	93	Yes
CW13032CC1	AD	APOE E4/E4	Female	100	100	99	100	100	99	97	100	99	97	Yes
CW13074AA1	AD	APOE E4/E4	Female	97	100	94	96	100	99	97	97	95	91	Yes
CW13098AA1	AD	APOE E4/E4	Female	89	100	93	81	100	99	95	100	78	98	Yes
CW13037AA1	AHN	ABCA7 G1527A	Male	98	100	96	80	100	98	95	96	98	96	Yes
CW13000AA1	AD	APOE E2/E4	Female	97	100	94	82	96	98	97	100	98	97	Yes

[00264] Levels of soluble TREM2 (sTREM2) protein secreted by microglia post thaw were quantified from conditioned media collected from microglia generated from a panel of iPSC donors using a Simple Step ELISA (AbCam) (FIG. 45). Microglia generated from apparently healthy normal donors and disease specific donors were thawed and plated at the same density in maturation media in a 96 well Primaria plate. The spent media was collected at Day 3 post thaw and day 7 post thaw. The cultures were half fed with fresh maturation media on day 3 and Day 5 post thaw.

[00265] The data revealed a difference in the levels of soluble TREM2 between various samples of microglia derived from donor exhibiting an R47H genotype revealed the highest level of Soluble TREM levels at day 3 post thaw and it stayed high even 7 days post thaw. Although this donor was asymptomatic and hence classified as AHN, the iPSC derived microglia secreted high levels of sTREM. This data is in agreement with the high levels sTREM2 observed in the cerebrospinal fluid of Alzheimer disease patients and associated with this mutation status (Cheng *et al.*, 2016). This data is a powerful example to use iPSC derived microglia in designing predictive kits of screening for the onset of neurodegenerative diseases. Young donors with an APOE4/4/ genotype exhibiting the onset of AD revealed a high level of soluble TREM compared to older donors with the same genotype. The presence of SNPs in CD33 or ABCA7 gene did not seem to enhance the release of soluble TREM in the supernatant media. AHN Donor 12068 revealed a high level of soluble TREM at 3- and 7-days post thaw.

[00266] Neuroinflammation contributes to progression and pathogenesis of many neurodegenerative diseases. Resident microglia and astrocytes in the brain release cytokines which can play both pro-inflammatory and anti-inflammatory roles in the brain depending on the stimulus and microenvironment. This fluctuation between the pro- and anti-inflammatory profiles has been associated with the onset of AD and other neurodegenerative diseases. To quantify the levels of cytokines and chemokines released by disease associated microglia (DAM), cryopreserved AHN and DAM microglia were thawed into MDM medium and plated on to Primaria 96 well plate at 50,000 cells/well. Cells were plated for three days prior to starting stimulations with 100 ng/ml LPS to check M1 mediated response or with 10ng/ml IL-4 along with 10uM dBu-cAMP to trigger an M2 specific response. Stimulations were performed in triplicate for 24 hours. Supernatant was spun down to remove cells and debris and immediately placed at -20°C. The supernatants were analyzed on a multiplex

Luminex assay. The results of this multiplex Luminex assay is captured as a heat map in FIG. 27C.

[00267] Chemokines CCL1, CCL2, CCL3, CCL4, CCL8, CCL11, CCL13,CCL17, CCL18, CCL20, CCL22, CCL24, serve as chemoattractants and mediate the recruitment of myeloid cells, granulocytes, lymphoid cells or neural precursor cells into inflamed areas, enhance phagocytic response and are generally upregulated in Alzheimer's Disease (AD) or Multiple Sclerosis. In response to LPS or dBu-cAMP, microglia derived from APOE E4/E4, AP0E E2/E4, TREM2 R47H, ABCA7 G1527A and CD33 (bearing the rs429358 SNP) derived microglia released a higher level of all these analytes compared to AHN lines. The fold increase varied from 0.1 to as high as 7 fold between various microglial genotypes. This data from disease associated microglia supports an earlier finding demonstrating an increase in CCL2 and CCL5 expression in AD brain samples. CCL2 expression in the brain and cerebrospinal fluid (CSF) has been reported as a reliable predictor of AD severity by Westin *et al.*

[00268] APOE E4/E4, AP0E E2/E4, TREM2 R47H, ABCA7 G1527A and CD33 (bearing the rs429358 SNP) derived microglia released marginally elevated levels of soluble CD163, a marker of inflammation and inflammatory diseases related to M2 polarization. The release of sCD163 prevents monocyte hyperactivation and reduces the secretion of pro-inflammatory cytokines TNF-alpha, IL-1beta, IL-6 and IL-8. A similar trend was observed with Chitinase-3 which plays a role in tissue remodeling during neural inflammation (Melief *et al.,* 2012); Minett *et al.,* 2016).

[00269] PD-L1 and its receptor, PD-1, elicit inhibitory signals that regulate the balance between T-cell activation, tolerance, and immune-mediated tissue damage. In response to LPS, APOE E4/E4 derived microglia had no increase compared to AHN derived microglia. TREM2 R47H, APOE E2/E4, and CD33 (bearing the rs429358 SNP) derived microglia displayed an increase compared to AHN lines. In response to IL-4 + dBu-cAMP, APOE E4/E4, TREM2 R47H, ABCA7 G1527A displayed a marginal increase over AHN lines, while APOE E2/E4 derived microglia had a 7-fold increase compared to AHN derived microglia.

[00270] APOE E4/E4, AP0E E2/E4, TREM2 R47H, ABCA7 G1527A and CD33 (bearing the rs429358 SNP) derived microglia released marginally elevated levels of soluble Fractalkine, a soluble chemokine promoting chemotaxis, survival and enhancing neuroprotection by decreasing levels of TNF-alpha and Nitic Oxide during neuroinflammation.

[00271] APOE E4/E4, AP0E E2/E4, and ABCA7 G1527A derived microglia released high levels of CXCL1/GRO alpha in response to both LPS and dBu-cAMP whereas TREM2 R47H and CD33 derived microglia revealed a marginal increase in the levels of this cytokine released, implying a correlation of this analyte with the APOE and ABCA7 genotypes. A recent study showed that CXCL1 could contribute to inflammation response in the development of AD, but not as a potential genetic factor conferring the predisposition to AD in the pathogenesis of this disease. Under physiological conditions CX3CR1 maintains microglial homeostasis by limiting their activation. The high levels of this cytokine released post stimulation indicates the onset of a rescue mechanism by CX3CR1 to preserve homeostatic function in disease associated microglia associated with APOE or ABCA7 G1527A genotypes. Alternately, the high levels of CX3CR1 secreted by APOE E4/E4 and ABCA7 G1527A activated microglia could be a signal to promote neuronal degeneration (Atagi *et al.,* 2015; Wolfe *et al.,* 2018).

[00272] APOE E4/E4 and AP0E E2/E4 derived microglia released high levels of IL-6 in response to both LPS and IL4/dBu-cAMP while all other genotypes secreted similar levels of IL-6 as AHN. IL-6 secretion attracts granulocytes, promotes a cell-mediated humoral Th2 response and trigger inflammation. This mechanism would again support greater neural inflammation associated with the APOE E4/E4 genotype.

[00273] ABCA7 G1527A derived microglia released high levels of IL-1 beta and IL-1 alpha in response to LPS, while the other genotypes secreted comparable levels to AHN microglia.

[00274] APOE E4/E4, AP0E E2/E4, and ABCA7 G1527A derived microglia also released high levels of IL-8/CXCL8 in response to LPS than dBu-cAMP. ABCA7 G1527A derived microglia released high levels of IL-8 implying the onset of a pro-inflammatory response contributing to brain injury.

[00275] Microglia also secrete proteolytic enzymes and matrix metalloproteinase that may eliminate Aβ deposition and limit AD process thus performing a neuroprotective role in AD. APOE E4/E4, AP0E E2/E4, TREM2 R47H, ABCA7 G1527A and CD33 (bearing the rs429358 SNP) derived microglia released marginally elevated levels of MMP-9 and MMP-12 compared to AHN derived microglia.

[00276] R47H TREM2 derived microglia released over 7 times more IL-12 p70 in comparison to AHN derived microglia when stimulated by LPS or M1 stimulation. On the other hand, AP0E E2/E4 released over 7 times more IL-12 p70 in comparison to AHN derived microglia in response to IL4+ dBu-cAMP or M2 stimulation. The other genotypes revealed a marginal increase in the levels of IL-12 secretion. Primary microglia produce IL-12 in the brain to control immune responses during infection or in Th1 cell-mediated autoimmune diseases of the CNS. The increased levels of IL-12 by R47H TREM2 derived microglia implies a strong activation of cytotoxic activity of NK-cells and T cells.

[00277] Finally, APOE E4/E4, AP0E E2/E4, ABCA7 G1527A derived microglia released similar or marginally high levels of IL-13, IL-18, IL-23 and Alpha Synuclein levels, implying a lack of correlation of these analytes with the above-mentioned genotypes.

[00278] Neuroinflammation is an important contributor to Alzheimer's disease (AD) pathogenesis and progression. A combination of several inflammatory mediators creates a unique signature associated with a particular SNP mutation. iPSC derived microglia from disease specific donors can be used to determine the key signature cytokines associated with various microglial SNPs and mutation associated genotypes.

[00279] The phagocytic function of microglia is important to preserve the neuroprotective effect. Microglia mediated phagocytosis can be impaired by disease specific SNPs or mutations which in turn can affect critical homeostatic mechanisms in the brain. The phagocytic function of Disease associated microglia (DAM) was evaluated in the presence of pHrodo labelled bacterial S aureus and amyloid beta to compare the role of disease associated SNPs on phagocytic function of microglia. This function can be used for high throughput screening applications.

[00280] Among these microglial expressed disease associated genes, sequence variants in the gene encoding the triggering receptor expressed on myeloid cells 2 (TREM2) and APO E isoforms are associated with an extraordinarily increased risk for AD. APOE is the primary cholesterol carrier in the brain, and plays an essential role in lipid trafficking, cholesterol homeostasis, and synaptic stability. Anti-ApoE immunotherapy inhibits amyloid accumulation and deposition further supporting a role of ApoE in Aβ aggregation and clearance. ApoE expression has been shown to be significantly upregulated in disease-associated microglia. ApoE4/E4 isoform has been shown to intrinsically affect microglia physiology by upregulating motility and phagocytic behavior in vitro. ApoE4/E4 overexpression has been shown to reduced uptake of Abeta in contrast to the other isoforms. The role of ApoE2, the third most common major ApoE isoform, in neurodegeneration has been shown to delay the onset of disease in familial AD. ApoE-isotype specific effects on iPSC derived microglia function have not been thoroughly investigated to date.

[00281] TREM2 senses lipids and mediates myelin phagocytosis. Loss-of-function (LOF) variants of TREM2, have been associated with an increased amyloid plaque seeding, reduced amyloid clustering and with an added interaction with ApoE triggers signaling cascade leading to reduced microglial clustering and ApoE accumulation in amyloid plaques with impairment of function.

[00282] The phagocytic function of Disease associated microglia (DAM) was evaluated in the presence of bacterial S. aureus and amyloid beta to compare the role of disease associated SNPs on phagocytic function of microglia.

[00283] On the same lines, ATP-binding cassette transporter A7 (ABCA7) has been identified as a susceptibility factor of late onset Alzheimer disease in genome-wide association studies. ABCA7 has been shown to mediate phagocytosis and affect membrane trafficking. ABCA7 is strongly associated with AD. Phagocytic clearance of amyloid-beta is impaired in Abca7-/- mice. iPSCs derived from patients a possessing a missense variant associated with G1527A substitution in ABCA7 provide BCA7 plays a role in the regulation of Abeta homeostasis in the brain to altered phagocyte function.

[00284] CD33 is an immunomodulatory receptor linked to Alzheimer's disease (AD) susceptibility via regulation of phagocytosis in microglia. TREM2 interacts downstream of CD33 in modulating microglial physiology and metabolism, thus iPSC derived microglia expressing WT and CD33 rs3865444 SNP can be used to validate the role of CD33 associated with impaired phagocytic function (Caldeira *et al.,* 2017).

[00285] Cryopreserved AHN and DAM microglia were thawed, cultured for three days in maturation media and exposed to pHrodo labeled Amyloid Beta and pHrodo S aureus. The kinetics of phagocytosis were measured using the IncuCyte Live-Cell Analysis System. The total red object integrated intensity was used to quantify the functional response.

[00286] TREM2 R47H microglia and ABCA7-G1527A microglia revealed a strong phagocytotic ability to bacterial S. Aureus compared to AHN microglia. CD33 (bearing the rs429358 SNP) microglia revealed comparable phagocytotic ability to bacterial S. Aureus compared to AHN microglia. TREM2 R47H microglia, ABCA7-G1527A microglia and CD33 (bearing the rs429358 SNP) microglia revealed reduced intensity of phagocytosis in the presence of Amyloid Beta in comparison to AHN microglia.

[00287] CW13030EE1 APOE 4/4 displayed the higher intensity of phagocytosis with S. aureus and Amyloid Beta compared to AHN microglia. CW13098AA1 APOE 4/4 displayed a lower intensity of phagocytosis with both S. aureus and Amyloid Beta compared to AHN microglia. CW13005AA1 APOE 2/4 microglia revealed strong phagocytic ability to Amyloid Beta and a reduced phagocytosis to S. aureus compared to AHN derived microglia. CW13074AA1 APOE 4/4 microglia exhibited a similar trend of phagocytosis for S. aureus as AHN microglia and a slightly enhanced phagocytosis to Amyloid Beta to AHN microglia cell lines.

[00288] A comprehensive understanding of the genetic alterations that target homeostatic, pro-inflammatory and anti-inflammatory microglial subtypes can provide novel biological insights and facilitate target prioritization for immunomodulatory therapeutic approaches for neurodegenerative diseases.

Example 9– Additional Characterization of Microglia

[00289] Extracellular nucleotides, such as ATP and ADP, are known to elicit receptor mediated pathways termed as the "purinergic signaling" pathway. Physiological processes such as tissue homeostasis, wound healing, neurodegeneration, immunity, inflammation and cancer are modulated by purinergic signaling. Extracellular ATP and P2 receptors are important for the microglial activation mechanism. P2X receptors are ionotrophic receptors that bind to ATP or their derivatives. One of the P2Y receptors is a G-protein coupled receptor that responds to ADP. Under pathological conditions, nucleotides such as ATP are released or leaked from injured cells and function as ‘‘find me’’ or “eat me” signals to evoke process extension, chemotaxis, and phagocytosis by microglia. P2 receptor activation also induces cytokine production from microglia, including interleukin-1b (IL-1b), interleukin-6

(IL-6), and tumor necrosis factor alpha (TNFα). Such pro-inflammatory mediators have been shown to dynamically change G protein coupled receptor (GPCR) expression and function in astrocytes.

[00290] The purinergic receptor response of microglia was characterized. Microglia were thawed in microglia differentiation media and reconstituted to a cell suspension of 200,000 cells/well. 15 µl of cell suspension of microglia was added per well of a 384 well plate. The cells were treated with different doses (0-1000nM) of BzATP and ADP. The response to BzATP and ADP was also measured in the presence of AZ11645373 (P_2X_7 antagonist) and AZD1283 (a potent antagonist of the $P2Y_{12}$ receptor). For all treatments 10 ul of a 4X stock of the compound or inhibitor was added to the cells. The cells were exposed to the compounds in the presence or absence of inhibitors for 30 minutes before the assay. One bottle of FLPR Calcium-6 was reconstituted to 11ml with Assay Buffer B and 15µl of the dye solution added to the cell suspension in the presence of the compounds. The cells were incubated at 37C for 1.5 hours and imaging was performed on the FDSS µCELL system.

[00291] FIG. 44A shows microglia with ATP/BzATP All Traces and FIG. 44B shows microglia with ATP/BzATP Sample Traces. FIGS. 44C-F show the response of microglia to BzATP, ADP, BzATP in the presence of P_2X_7 antagonist AZ11645373, and BzATP in the presence of P_2X_7 antagonist A438079 FIG. 44G shows a dose dependent response to demonstrate functional ADP dependent response in microglia in the presence of AZD1283.

Example 10– Generation of Neural Precursor cells from iPSC

[00292] The successful development of *in vitro* disease models is dependent on the availability of large quantities of end stage lineages derived from patient derived iPSCs. Neural presursor cells (NPCs) are self-renewing progenitors with the ability to generate neurons and glia (Breunig et al., 2011). There are many established protocols with varying efficiencies for generating NPCs from primary neural cells and iPSCs (Shi *et al.*, 2012a, Shi *et al.*, 2012b). Most of the recent protocols rely on the inhibition of the SMAD signaling pathway. The present application describes a simple protocol to generate NPCs across different iPSCs lines utilizing the spontaneous drift of iPSC towards ectoderm without using the dual SMAD inhibition pathway. The rationale to generate this cell type is to pair it with iPSC derived microglia to generate long term co-culture assays to mimic human brain development and complex intercellular interactions between neural lineages, microglia, endothelial cells, pericytes, and astrocytes in a dish derived from normal and disease specific iPSC cells.

[00293] In this study, multiple episomally reprogrammed iPSC lines were maintained on MATRIGEL™, Laminin, or Vitronectin coated plates and E8 media. The iPSCs were maintained under hypoxic conditions before the onset of differentiation to generate neural precursor cells. To initiate neural precursor differentiation, iPSCs were harvested and seeded at 15K/cm^2 on MATRIGEL™, Laminin or Vitronectin plates using E8 media in the presence of rock inhibitor. The cells were placed in fresh E8 media for the next 48 hours in the absence of rock inhibitor. The next step involved the preconditioning step comprising placing iPSC cultures in DMEMF12 media supplemented with 3uM CHIR for 72hours with a daily change in media under normoxic conditions. Cells were harvested at the end of the preconditioning step and either replated back in a 2D format on MATRIGEL™, Laminin or Vitronectin plates at 30K/cm^2 or generated 3D aggregates using Ultra low attachment plates or spinner flasks at a density of 0.3 million cells per ml in the presence of a rock inhibitor. The cultures were fed every other day with E6 media supplemented with N2 for the next 8 days under normoxic conditions. On day 14 of differentiation the cultures were harvested and individualized using TrypLE. The cells were stained for the presence of Tra-162, CD56, CD15 by cell surface staining for flowcytometry and for the presence of Sox1, Nestin, β3 Microglobulin, and Pax-6 expression by intracellular staining for flow cytometry. The different steps involved in the generation of NPCs are outlined in FIG. 45A. The emergence of NPC markers at different days of differentiation across three iPSC lines is summarized in FIG. 45B. CD56 was used as the marker for NPCs derived by this method. The cells were cryopreserved using CS10 and they retained purity and proliferation potential post thaw. NPCs were placed in downstream differentiation protocols to generate Astrocytes and Pan neurons.

[00294] Astrocytes were differentiated from NPCs according to the protocol outlined by Julia *et al.* Day 14 NPC cells were plated onto MATRIGEL™ coated 6 well plates at 15k/cm 2 in Science Cell Astrocyte Medium. The plates were given a full media exchange every two days. Every 6 days, or when cultures were ~90% confluent, the plates were harvested using Accumax and replated onto MATRIGEL™ coated 6 well plates at 15k/cm^2. The cultures were fed and replated as described above for 4 passages. At the end of 4 passages the culture was stained for surface markers, CD44 and glutamate aspartate transporter (GLAST), and intracellular markers, Glial fibrillary acidic protein (GFAP), Excitatory amino acid transporter

1 (EAAT1), Glutamine Synthetase (GS), Aquaporin 4 (AQP4), and S100 calcium-binding protein B (S100β) (FIG. 45C).

[00295] Cortical glutamatergic neurons were generated from NPCs using the protocol developed by Slosarek *et al* day 14 NPC were plated in E6 medium supplemented with 1 μM cyclic AMP, 10 ng/ml brain-derived neurotrophic factor (BDNF), and 10 ng/ml glial-derived neurotrophic factor (GDNF)) for 30 days. The medium was subsequently changed to cortical neural differentiation medium (E6 medium, 1 μM cyclic AMP, 10 ng/ml BDNF, 10 ng/ml GDNF, 100 ng/ml Insulin-like growth factor-I, and 2% B27 supplement) for an additional 30 days (Brennand *et al.*, 2011). Cortical glutamatergic neurons were observed between day 14-36 for different iPSC lines. The purity of the neural cultures was confirmed by staining for the presence of β3 Tubulin, MAP2 expression.

Example 11 – Triculture comprising neurons, microglia and astrocytes

[00296] The tri-culture was set-up by first maturing the iCell microglia. Cryopreserved microglia were thawed in an ultra-low attachment (ULA) 6 well plate for three days prior to tri-culture. The cells were placed in the presence of Microglia Maturation Medium containing IL-34 (100 ng/mL; Peprotech / 200-34), TGFβ1 (50 ng/mL; R&D Systems / 240-B), M-CSF (25 ng/mL; Peprotech / 300-25), CD200 (100 ng/mL; Acro Biosystems / OX2-H5228) and Fractalkine (100 ng/mL; Peprotech / 300-31).

[00297] Microglia Maturation Media

Material	Supplier/ Catalog #	Unit per Liter	Final Conc.
DMEM/F-12, HEPES, no phenol red	ThermoFisher/11039021	940mL	94%
N2	ThermoFisher/17502048	5mL	0.5%
B27 with RA	ThermoFisher/17504044	10mL	1%
10% BSA (in PBS)	Sigma/A1470	5mL	0.05%
MTG (11.5M)	Sigma/M6145	40uL	450uM
Ascorbic Acid (20mg/mL)	Wako/013-19641	2.5mL	50ug/mL
Pen/Strep GlutaMAX	ThermoFisher/15140 ThermoFisher/35050	10mL 10mL	1% 1%
NEAA	ThermoFisher/11140050	10mL	1%
Insulin-Transferrin-Selenium (ITS-G)(100x)	ThermoFisher/41400045	10mL	1%
Insulin	Sigma/I9278	500uL	5ug/mL
MCSF (100ug/mL)	Peprotech/300-25	250uL	25ng/mL
TGF-β1 (100ug/mL)	R&D Systems/240-B	0.5mL	50ng/mL

IL-34 (100ug/mL)	Peprotech/200-34	1mL	100ng/mL
CD200 (100ug/mL)	Acro / OX2-H5228	1mL	100ng/mL
CX3CR (100ug/mL)	Peprotech / 300-31	1mL	100ng/mL

[00298] CD200, also known as OX-2 membrane glycoprotein (OX-2), is a type-1 membrane glycoprotein, which contains two immunoglobulin domains (1 Ig-like C2-type (immunoglobulin-like) domain and Ig-like V-type (immunoglobulin-like) domain), and thus belongs to the immunoglobulin superfamily. CD200 / OX-2 is widely expressed in multiple cell types. CD200 interacts with a structurally related receptor (CD200R) expressed mainly on myeloid cells and is involved in regulation of macrophage and mast cell function. CD200 also play a role in prevention of graft rejection, autoimmune diseases and spontaneous abortion.

[00299] Fractalkine is a CX3CL chemokine that signals through the CX3CR1 receptor. Fractalkine has been shown to chemoattract monocytes, microglia cells and NK cells. Fractalkine is, at this time, the only CXC3C chemokine that contains three amino acid residues between the first and second cysteine residues of the chemokine domain.

[00300] 3 post thaw the microglia cultures were harvested, washed and the cell pellet was diluted in the tri-culture medium (Table 6) to obtain 7,500 cells / 70uL / well of a 96 well plate. The cells may be seeded at a density of 7,500 cells/well of a 96 well plate.

[00301] Table 6: Composition of tri-culture media

Tri-Culture Medium	
Material	**Final Concentration**
Neurobasal™ Medium	100%
B27 with RA	1%
GlutaMAX	1%
M-CSF	25 ng/mL
IL-34	100 ng/mL
TGF-β1	50 ng/mL

[00302] Cryopreserved GABA neurons were thawed and resuspended to obtain 50,000 cells / 70uL / well of a 96 well plate in tri-culture medium. The neurons may be seeded at a density of 50,000 cells/well of a 96 well plate. Similarly, cryopreserved astrocytes were thawed and diluted to a density of to obtain 8,000 cells / 70uL / well of a 96 well plate in tri-culture medium. The astrocytes may be seeded at a density of 8,000 cells/ 70uL/well of a 96 well plate.

[00303] On the day prior to setting up the coculture, the plates were coated with PEI/Geltrex solution (PEI at a concentration of 0.07% and Geltrex at a concentration of 12-18 ng/mL) and rinsed prior to plating the cells of interest. All of the cell types were plated to generate mono, bi or tri-cultures. On the day of plating, 70uL of microglia, 70uL of GABA neurons, and 70uL of iCell astrocytes were each adjusted to the density mentioned above and plated on to each well. The cells were spread by slowly shaking the plate on a flat surface and cultured at 37°C, 5% CO2. The cells half fed with tri-culture medium from the plate every 4 days. A combination of monocultures of neurons, astrocytes and microglia and bi-cultures with neurons and astrocytes, or astrocytes and microglia or neurons and microglia were also generated at the same density. Mono-, bi- and tri-cultures were maintained in the triculture medium and the precise ratio of cell types for 14 days before performing end stage staining or assays. A schematic description of this set up is described in (FIG. 66).

[00304] 14-day cultures were stimulated with LPS for 24hrs and the cultures with and without stimulation as well as the mono-culture and bi-culture combinations were fixed and stained to detect the presence of solution was prepared using Pan Neuronal Marker (1:1500; Millipore, Catalog: MAB2300), Anti-Iba1 (1:500; Wako Chemicals, Catalog: 019-19741), and Anti-GFAP (1:500; Abcam, Catalog: ab4674). Images of mono-culture (FIG. 50A), bi-culture (FIG. 50B) and tri-culture (FIG. 50C), a collage of images in tri-culture media with or without the microglia factors (TGFβ, IL-34 and MSCF) is shown (FIG. 50D) and finally a high resolution image of the tri-culture is shown (FIG. 50E). All images were captured using the ImageXpress Micro Confocal High-Content Imaging System (Molecular Devices). The quantification of the surviving cells in the tri-culture was performed by FUJIFILM AI-based image analysis software (FIGS. 51A-51B)

[00305] The triculture system of microglia, astrocytes and neurons were tested for disease-associated differences by substituting the apparently healthy microglia with

TREM2HZ and TREM2HO microglia. A comparative analysis of various cytokines and chemokines released by the mono-, bi-, and tri-culture combinations was assessed in the presence and absence of LPS stimulation. A schematic representation of the experimental design is depicted in (FIG. 52).

[00306] The coculture system was maintained for 14 days prior to starting stimulation with 1 ug/mL LPS. Stimulations were performed in duplicate for 24 hours. Supernatants were spun down to remove cells and debris and immediately placed at -20°C. The supernatants were analyzed in triplicate via a multiplex Luminex assay

[00307] The results to the Luminex assay are represented as a heatmap in FIGS. 53-56. The compilation of cytokines released by reactive astrocytes (CX3CL1) and non-reactive astrocytes (FGF2) is summarized in FIG. 53. The compilation of all the M1 factors released in the media (TNF alpha, IL-6, CCL2, CCL3, CCL4, IL-1 beta, IL-12, IL-13, IL-8, Interferon gamma, IL1-Alpham FAS ligand, IL-2, GMCSF, Granzyme B, ICAM-1, CXCL11) is summarized in FIG. 54. The compilation of M2 (anti-inflammatory) factors (IL-4, IL-10, IL-21, VEGF, CCL5, IL-17, IL1-RII, GCSG, CXCL5) is summarized in FIG. 55. The release of the C3 complement system in summarized in FIG. 56.

[00308] Microglia impact neuronal network activity on the microelectrode array (MEA). Synchronously bursting co-cultures of Gluta neurons and astrocytes (cell ratios ranging from 4:1 to 6:1) were established after 2 weeks in BrainPhys medium supplemented with B27, NSS, N2, and laminin. Subsequent addition of microglia to the existing culture at Week 2, Week 3, Week 4 or beyond, in cell numbers similar to astrocytes (MGL:ASC ratios ranging from 0.25:1 to 1:1) resulted in altered network bursting architecture that was visualized and quantified on the MEA. The impact of microglia can be short-lived or long-lasting and can be strongly influenced by medium and supplements, thereby offering a range of strategies to improve the neural network (FIG. 57).

[00309] A 3D or 2D culture of microglia, astrocytes and glutaneurons may be cultured in BrainPhys neuronal media supplemented with B27 containing vitamin A, 0.5 mM glutamax N2 Plus media supplement, 460 µM thioglycerol, 1X insulin-transferrin-selenium, 5.4 µg/ml human insulin solution 10-25 ng/ml BDNF, 10-25 ng/ml GDNF, 1 mM creatine, 200 nM L-ascorbic acid, 1 µg/ml laminin or fibronectin, 5-15 ng/ml TGF- b, 100 ng/ml human IL-

34, 100 ng/ml human M-CSF, 1.5 µg/ml cholesterol, 1 ng/ml 9 or 11 eicosenoic acids, and 100 ng/ml oleic acid or linolenic acid.

[00310] Microglia demonstrate robust phagocytosis of various substrates (bioparticles, amyloid beta, Tau, synaptosomes, etc.) when in mono-culture under standard conditions. Panels of iPSC-derived microglia (including TREM2 HZ and TREM2 HO) for disease modeling yielded different features and kinetics in a phagocytosis assay. Creation of more complex bi-culture and tri-cultures with GABA- or glutaneurons and astrocytes resulted in enhanced phagocytic activity (FIG. 58). This platform approach can be used investigate the different mechanisms of TREM2 variants on microglia biology, phenotype, and function.

[00311] 3D triculture spheroid formation with astrocytes, microglia and Gluta neurons was done to study the role of microglia and astrocytes on neuronal MEA function. Cryopreserved microglia, astrocyte and Gluta neurons were thawed into NB complete media (NB+Glutamax+B27-VitA+NSS) supplemented with IL-34 and MCSF. A wide range of ratios among the three cell types were tested and the representative images shown in FIG. 59 were generated using a ratio of 5k:5k:30k (Microglia: Astrocytes: GlutaNeurons). The spheroids were formed in S-Bio V bottom 96well plates in NB complete media plus IL-34 and MCSF. 48 hours later the media was switched to Brainphys complete media (Brainphys+N2+B27-vitA+NSS) to maintain the culture for 2 weeks.

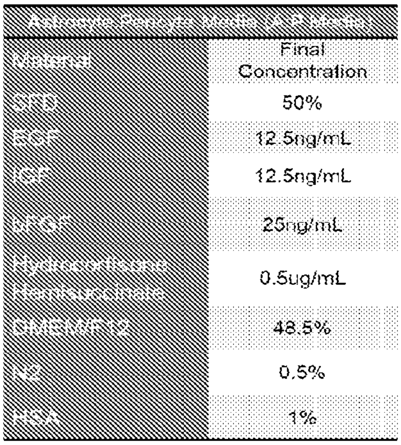
[00312] Functional assessment of calcium transients was performed in tri-culture spheroids at Day7 and Day15 in the presence of all three cell types. Calcium transients were measured using EarlyTox calcium dye (Molecular Devices) and recorded on the CLARIOstar instrument. Robust spontaneous calcium oscillations were detected in the tri-culture spheroid as early as Day7 post assembly, revealing the presence of a fully functional neuron network as shown in FIG. 60. Calcium oscillations may be detected by fluorescent dyes that change emissions due to voltage of calcium concentrations. Instruments that can detect this are FDSS, Flipper, or time lapse confocal imaging.

Example 12– Setting up a blood brain barrier model using cryopreserved BMECs, pericytes, and astrocytes

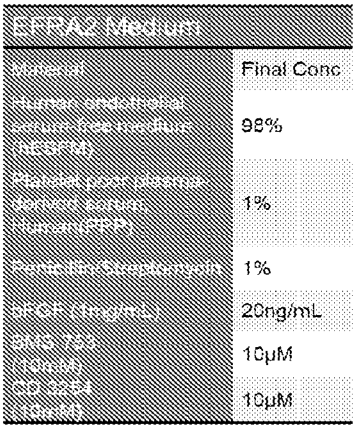
[00313] A blood brain barrier (BBB) model was generated using cryopreserved iPSC-derived BMECs, pericytes and astrocytes. The sequential steps involved in setting-up the sandwich BBB at the specific ratios of all three cell types is depicted in FIG. 61. The details of the set-up are described as follows: Apical (blood side) of transwell was coated with collagen-IV (200-400 µg/mL)/fibronectin (50-100 µg/mL) and left overnight at 4°C or incubated for 4 hours at 37°C. The entire plate, with lid, was inverted and the base of plate was removed without disturbing the transwells. The basolateral (brain side) of the inverted transwell, was coated using 0.1% gelatin for 30-60 minutes at room temperature. The base of the plate was placed back on using spacers to allow the coated basolateral side of the transwell to remain undisturbed. The tranwells were covered to reduce evaporation of apical and basolateral coated sides. Astrocytes and pericytes were thawed into IMDM media containing 10% FBS and spun at 600g for 8 minutes. Cells were then counted and combined at a 1:2 ratio in Astrocyte:Pericyte Media (A:P Media) (Table 7) containing Y-27632. Astrocytes were plated at 333,333 cells/cm^2 and pericytes were plated at 666,666 cells/cm^2. 0.1% gelatin was removed from the inverted basolateral side of the transwell and the combined cell suspension was plated on the inverted basolateral side of transwell in 100-170 µL per transwell. The base of the plate was placed back on spacers over the inverted transwells and the plate was incubated in normoxic atmosphere (5% O2) for 4 hours. Next, the plate was placed in BSC and the base of the plate and spacers are removed. The base of the plate was then returned and the plate was flipped to the original orientation. The collagen/fibronectin was removed from the apical side of the transwell and each well was given an addition of 300 µL to the apical side of the transwell and 1 mL to well of A:P media. The plate was then incubated in normoxic atmosphere at 37°C overnight. BMECs were thawed and placed at a 1:1 ratio in EFRA2 + Y-27632. A:P media was removed from the apical side of the transwell and BMECs were seeded at 1.3 X 10^6/cm^2 onto the apical side of the transwell in 300 µL EFRA2 + Y-27632. The media in the well was aspirated and 1mL EFRA2 + Y-27632 was added. The plate was placed in normoxic atmosphere at 37°C.

[00314] FIG. 62 depicts the TEER function of cryopreserved BMECs thawed and plated at days 3-8 post thaw onto FN/ColIV-coated Corning transwell inserts at 1.3 x10^6 cells/cm^2 in BMEC media. FIG. 63 depicts the TEER function of cryopreserved BMECs and pericytes. FIG. 64 depicts the TEER function of cryopreserved BMECs and astrocytes and finally FIG. 65 depicts the TEER function of cryopreserved BMECs, pericytes and astrocytes. The ratios of all the cell types used to set up the tri-culture is described in Table 8.

[00315] Table 7. Astrocye:pericyte media and EFRA2 media composition.

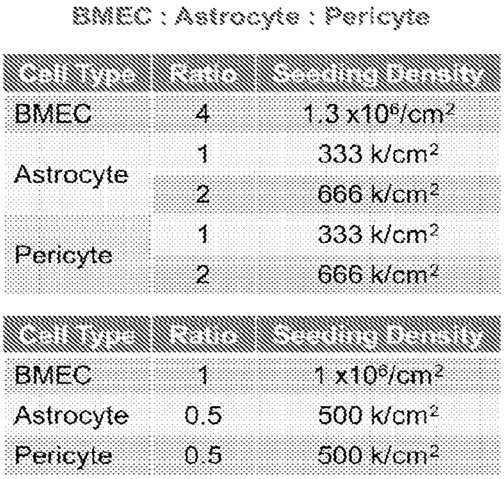


Astrocyte Pericyte Media (A-P Media)	
Material	Final Concentration
SFD	50%
EGF	12.5ng/mL
IGF	12.5ng/mL
bFGF	25ng/mL
Hydrocortisone Hemisuccinate	0.5ug/mL
DMEM/F12	48.5%
N2	0.5%
HSA	1%



EFRA2 Medium	
Material	Final Conc
Human endothelial serum-free medium (hESFM)	98%
Platelet poor plasma-derived serum, Human(PPP)	1%
Penicillin/Streptomycin	1%
bFGF (1mg/mL)	20ng/mL
BMS 753 (10mM)	10μM
CD 2314 (10mM)	10μM

[00316] Table 8. Cell ratios to set up the blood brain barrier.



BMEC : Astrocyte : Pericyte

Cell Type	Ratio	Seeding Density
BMEC	4	$1.3 \times 10^6/cm^2$
Astrocyte	1	333 k/cm^2
	2	666 k/cm^2
Pericyte	1	333 k/cm^2
	2	666 k/cm^2

Cell Type	Ratio	Seeding Density
BMEC	1	$1 \times 10^6/cm^2$
Astrocyte	0.5	500 k/cm^2
Pericyte	0.5	500 k/cm^2

Example 13 – Characterization of role of TREM2 in microglia

[00317] GWAS in AD identified TREM2 as a key modulator of AD risk. Heterozygous mutations in TREM2 lead to increased AD risk whereas homozygous mutations lead to the neurological condition known as Nasu-Hakola Disease. Therefore, while in each case the CNS is affected, the pathobiology and the clinical manifestations of a heterozygous versus homozygous mutation are distinct. To better understand the role of TREM2 mutations on AD risk, heterozygous TREM2 loss of function microglia were generated. Validation of partial loss of TREM2 microglia identified pathways unique to HetTREM2 KO supporting the notion that heterozygous TREM2 KO microglia better model the phenotype that arises from the inheritance of TREM2 mutations that lead to increased AD risk.

[00318] Use of heterozygous TREM2 LOF microglia to identify druggable pathways for AD. Heterozygous mutations in TREM2 lead to AD but homozygosity leads to Nasu-Hakola Disease. Examination of the HZ and HO TREM2 KO lines in comparison to AHN isogenic microglia identified unique pathways altered by partial loss of TREM2 function that was less apparent or absent in complete loss of TREM2 function microglia mutants. For example, gene expression of the master transcriptional regulator of cholesterol biosynthesis, SREBF2 was significantly reduced resulting in concomitant reduction of cholesterol biosynthetic enzyme genes, suggesting down-regulation of cholesterol synthesis due to partial but not complete loss of TREM2 function (FIG. 67). In addition, the phenotype of HZ TREM2 identified a reprogramming of fatty acid metabolism/catabolism indicating that TREM2 signaling plays an essential role in regulating lipid homeostasis in microglia. Therefore, unique pathways were uncovered only in the context of TREM2 partial loss of function that can serve as novel therapeutic targets to restore microglia function and homeostasis in AD and other neurological diseases.

[00319] Targeting pathways identified through heterozygous loss of TREM2 function to improve liver function, lung function, vision loss and reduce atherosclerosis. Given that TREM2 is also expressed in other tissue resident macrophages (lung alveolar macrophages, Kupffer cells, sub-retinal microglia), the generation of the hetTREM2 KO identified that TREM2 signaling modulates lipid homeostasis and that TREM2 can serve as a therapeutic target to restore lipid dysfunction in different tissue resident macrophages that play a role in the etiology of a multitude of chronic diseases.

[00320] Targeting TREM2 function to enhance interferon signaling without down-regulating cholesterol biosynthesis. In addition, an interferon signature was identified. Recent studies indicate a link between interferon signaling and cholesterol biosynthesis. Thus, the interferon axis can also be targeted to modulate cholesterol biosynthesis in the context of tissue resident macrophages to restore homeostatic function.

[00321] Targeting the Gas6-Axl axis via TREM2. Examination of HZ and HO TREM2 microglia identified a dose response on the gene expression of the Gas6/Axl axis (FIG. 68). The Axl signaling axis is associated with disease associated microglia that play a role in responding to the neurodegenerative cascade in AD and other neurological diseases (Krasemann *et al.*, 2018). Therefore, TREM2 activation enhances Gas6/Axl signaling and enhances the DAM phenotype. However, the Gas6/Axl pathway also plays a role in the tumor microenvironment in supporting an immunosuppressive environment. Thus, antagonizing TREM2 and downregulating Gas6/Axl can enhance immune cell recruitment (Tanaka and Siemann, 2020).

[00322] Activation of Siglec-11 via TREM2 is neuroprotective. Use of HZ TREM2 microglia identified downregulation of Siglec 11, a CD33-related protein that prevents microglia neurotoxicity (Wang and Neumann, 2010), as a specific gene afflicted by partial but not complete TREM2 loss (FIG. 69). The TREM2-Siglec11 axis was only apparent in the HZ TREM2 microglia. Therefore, activation of TREM2 can be neuroprotective via upregulation of microglia Siglec-11.

[00323] Targeting TREM2 to restore GRN function in FTD. HZ and HO LOF microglia demonstrated a downregulation of GRN due to loss of TREM2 signaling (FIG. 70). Thus, activation of TREM2 can enhance GRN levels and restore the GRN loss of function phenotype in FTD.

[00324] Effect of TREM2 on Ion channels. The dose-dependent increase in gamma-aminobutyric acid receptor subunit epsilon (GABRE) by TREM2HZ and TREM2HO can activate microglia to release interleukin-6 and interleukin-12p40.

[00325] The enhanced expression of zinc-activated ligand-gated ion channel of the cysteine-loop superfamily of ligand-gated ion channels by TREM2 implies a direct link between TREM2 and expression of ion channels in generating a relevant functional model for neuroinflammation.

[00326] G-protein-coupled receptors (GPCRs) are one of the most frequently targeted receptors for developing novel therapeutics for central nervous system (CNS) disorders. As new functions for GPCRs are discovered, especially in the case of the 100 orphan GPCRs for which no endogenous ligand or clearly defined function are currently known, the number of drugs targeting GPCRs is expected to increase.

[00327] Microglia lacking TREM2 undergo global changes in their metabolism, resulting in reduced ATP levels and signs of stress and death. Given that a large percentage of today's pharmaceuticals target GPCRs, and TREM2 induces changes in many GPCRs imply that TREM2 may be a ligand as well as a regulator of microglial survival and function. GPCRs were found to be regulated by TREM2 which can be used for drug targeting applications.

Perturbations in TREM2 expression downregulated ADGRD1, ADGRE3, ADGRE5, ADGRG1, ADGRG3, ADORA2B, ADRB1, ADRB2, C5AR1, C5AR2, CCR2, CXCR2, CXCR4, EDNRA, FPR3, FZD1, GPBAR1, GPR157, LTB4R, LTB4R2, P2RX1, P2RY1, P2RY12, PTGER4 and SUCNR1. TREM2 expression downregulated expression of AVPR2, CNR2, GPR18, GPR84 and LPAR6.

[00328] Down regulation of TREM2 resulted in a dose-dependent reduction in expression of P2XRX1, P2RY12, and P2RY10 which function as ligand-gated ion channels with high calcium permeability. The decrease in P2RX and P2RY levels driven by TREM2 supports the role of TREM2 in age-associated changes in human microglia like cell adhesion and axonal guidance regulated by calcium signaling.

[00329] Effect of TREM2 on Transport Proteins. ATP-binding cassette (ABC) transporters are membrane-bound proteins that actively translocate endo- and xeno- biotic solutes across cellular membranes at the expense of energy by ATP hydrolysis, often against a concentration gradient. ABC transporters were discovered as proteins in the process of production, degradation and clearance of Aβ proteins. Specific transport proteins were found to be regulated by TREM2 which can regulate metabolism, phagocytic function and play a role in generating DAM (Disease Associated Microglia).

[00330] TP6ViG2 is a multisubunit enzyme that mediates acidification of intracellular compartments of eukaryotic cells to perform uptake and sorting function. The downregulation of ATP6ViG2 by TREM2 HZ and TREM2 HO lines supports the role of TREM2 on protein sorting, zymogen activation, receptor-mediated endocytosis, and synaptic vesicle proton gradient generation

[00331] ATP8A1 catalyzes the hydrolysis of ATP coupled to the transport of aminophospholipids from the outer to the inner leaflet of various membranes and ensures the maintenance of asymmetric distribution of phospholipids. Downregulation of ATP8A1 in TREM2 HZ and TREM2 HO lines supports the role of TREM2 on metabolism and migration of microglia function.

[00332] The solute carrier (SLC) group of membrane transport proteins includes over 400 members organized into 66 families located in the cell membrane. SLC transporters play a role in the termination of synaptic transmission for amino acid neurotransmitters in addition to their role in providing essential nutrients and osmolytes to neurons and glial cells.

SLC family members that were found to be affected by perturbations of TREM2 include ABCA3, ABCA7, ABCG1, ATP10A, ATP13A1, ATP13A2, ATP2A3, ATP6V1E2, ATP8A1, EPB41, SLC10A3, SLC15A3, SLC16A1, SLC16A3, SLC18A2, SLC19A1, SLC22A23, SLC25A1, SLC25A23, SLC25A4, SLC25A45, SLC26A11, SLC29A2, SLC29A3, SLC2A1, SLC2A5, SLC37A1, SLC37A2, SLC38A1, SLC38A5, SLC40A1, SLC41A2, SLC43A3, SLC44A2, SLC45A3, SLC47A1, SLC6A12, SLC6A8, SLC7A1, SLC7A5, SLCO4C1, and SPNS3. Perturbation of TREM2 also upregulated expression of transporters SLC11A1, ABCC5, SLC25A20, SLC35E3, ABCA1, SLC25A27,SLC15A2 and SLC25A42.

[00333] Many of these transported proteins regulate pH and acidify intracellular compartments, facilitate monocarboxylate transport and enhance transport of pharmaceuticals, toxins, hormones, increase permeability of the mitochondria. The data confirms the expression of a diverse array of transporters on microglia that shape physiological and survival functions. The effect of TREM2 on downregulating or upregulating a subset of transporters unveils their collaborative role in survival, phagocytic function and onset neurodegenerative diseases.

[00334] TREM 2 downregulated the expression of many key proteins that regulate the onset of neural inflammation. TREM2 downregulated ACVR1B, ACVRL1.AXL.CIITA CSF2RBCSF3R, EPHB6, IL11RA, IL17RA, IL21R, IL27RA, IL6R, IL6ST, IL7R, ITGAM, ITGAX, ITGB3, LMTK3, NLRP1, TLR3, TLR5, TNFRSF11A, TNFRSF12A, TNFRSF21, TNFRSF25, and TNFRSF9. TREM2 downregulation enhanced the expression of IL21R, ITGA2B, ITGA7, RYK, TLR2, TNFRSF10C, and FAS. Many of these molecules can be used in the diagnostic kits for detecting neural degeneration.

[00335] TREM2 downregulated the expression of the following enzymes expressed in microglia: LIPG, INPP5A, PADI4, GRK5. PCSK5, NEK2, ACE, PTK2, TSSK6, MMP19, SMPD3, PDE3B, PDE6G PRSS8, TPSAB1, PRKACB, DAGLA, QPCT, CDK14, ACE2, CPA3, NT5M, PIK3R6, RPS6KA5, PCSK4, TTK, HDC, CAMKK1, INPP1, MAPK12, LPIN1, IRAK2, PLK2, PDE6B, NAT8L, PROC, SPHK1, HPGD, CIT, INPP5E, CDK18, PTGS2,CASK, PLK1, FAAH,BCR, HDAC4, DMPK, ITPKB, ADA, CES1, MVK, TESK2, PIK3CA,TGM2, CFD, BUB1B, DPEP2, ENPP2, CYP27A1, ULK2, PIK3CB. SQLEKMT5C, AURKB, MAPK13, MAP3K12, TOP2A, PKMYT1, HMGCS1, ACAT2, DNMT3A, RRM2, CYP51A1, MGLL, KMT5A, BACE1, PIK3CG, TRIB1. PIK3R2, FASN, MMP2, ADCK2, LSS, GRK6, HMGCR, SRC, ACSS2, STK38L, FGR, IDI1, KDM5C, ABHD2, KSR1, LTC4S, FURIN, MVD, PRKAR2A, RPS6KA4, DAPK1, MPST, FDFT1, ADAM10, DDAH2, LGMN, PKN1, FDPS, SGK1, GAA, CPM, ALDH2, ASAH1, and CTSD.

[00336] TREM2 upregulated the expression of the following enzymes expressed in microglia: MMP9, CASP5, LIMK2, PLA2G4C, RAB27A, CFB, CYP2R1, CTSK, NUDT7, PRKY, ANPEP, SMPDL3A, PLA2G4B, STK32B, and PDK4.

[00337] TREM2 downregulated the expression of the following proteins expressed in microglia: BCL2, BIRC5, BIRC7, BRPF3, CD1D, CD22, CD276, CD37, CD6, CD74, CRY1, EPAS1, FABP3, FCGR3A, FCMR, HSPA1B, ILDR2, KIF11, LILRA4, NOTCH4, RBP1, RBP4, RBP7, RGS1, RGS2, RGS3, TACSTD2, TUBA1A and XIAP. TREM2 upregulated the expression of the following proteins expressed in microglia: CD14, CD36, CD80, CLEC4E, FCER1G, LAG3, LY96, PVRIG, RBP5, and SLAMF7. The present studies show the first direct link between perturbation/ downregulation of TREM2 associated with downregulation of COMT, NRXN2 and SST expression.

[00338] The COMT gene codes for an enzyme called catechol-O-methyltransferase that regulates personality, planning, inhibition of behaviors, abstract thinking, emotion, and working (short-term) memory. COMT catalyzes the O-methylation and inactivates catecholamine neurotransmitters, catechol hormones and shortens the biological half-lives of neuroactive drugs, like L-DOPA, alpha-methyl DOPA and isoproterenol. The levels of COMT in sporadic AD are highly variable. The genetic risk score based on the accumulation of multiple risk alleles in BDNF, COMT and APOE for AD has been used to predict late-life cognitive impairment in AD (Wollam *et al.*, 2015). NRXN2 belongs to the family of Nurexins that function as cell adhesion molecules and receptors. Deletion NRXN2 in mice lead to autism-related behaviors. The hormone Somatostatin (SST) controls rates of neurotransmission and proliferation of cells in the CNS.

[00339] In addition to COMT, NRXN2 and SST, TREM2HZ microglia additionally down regulated aldehyde dehydrogenase (ALDH1A2) that catalyzes the synthesis of retinoic acid (RA) from retinaldehyde. Homeobox 3 (HOXB3) a nuclear protein with a homeobox DNA-binding domain that is involved in development. Insulin like growth factor binding protein 2 (IGFBP2) that regulates neuronal plasticity to modulate high-level cognitive operations such as spatial learning and memory and information processing and Proteinase 3

(PRTN3) a serine protease that degrades elastin, fibronectin, laminin, vitronectin, and collagen types I, III, and IV and facilitates transmigration.

[00340] In addition to COMT, NRXN2 and SST, TREM2HO microglia additionally down regulated Ankyrins (ANK1) that links the integral membrane proteins to the underlying spectrin-actin cytoskeleton and play key roles in activities such as cell motility, activation, proliferation and maintenance of specialized membrane domains. ANKI interacts with the interferon signaling pathway. Pregnancy up-regulated nonubiquitous CaM kinase (PNCK) is a unique member of the calmodulin kinase family. PNCK is predominantly expressed in the central nervous system. It phosphorylates CREB1 and SYN1/synapsin and triggers signaling pathway that activate microglia. CaMKII is dysregulated in AD and this dysregulation is a key contributor to synaptic degeneration, NFT formation and memory deficits. The downregulation of PNCK by TREM2 suggests a role of PNCK in microglia proliferation and activation. Other genes that are downregulated include tubulin beta-4a chains (TUBB4) that bind GFP and nucleotides, sterile alpha motif domain containing 11 (SAMD11) and B9 domain-containing protein (B9D1). These genes function cell adhesion, proliferation, migration function of microglia. The downregulation of the listed genes along with TREM2 may be a contributing factor to the onset of AD.

* * *

[00341] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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WHAT IS CLAIMED IS:

1. A cell culture comprising induced pluripotent stem cell (iPSC)-derived microglia, astrocytes, and/or neurons in media.

2. The culture of claim 1, wherein the culture comprises iPSC-derived microglia, astrocytes, and neurons.

3. The culture of claim 1, wherein the cell culture is further defined as a triculture.

4. The culture of claim 1, wherein the neurons are excitatory neurons or inhibitory neurons.

5. The culture of any of claims 1 -4, wherein the neurons are gabaergic neurons, dopaminergic neurons, or glutamatergic neurons.

6. The culture of any of claims 1-5, wherein the microglia are derived from isogenically engineered iPSC lines.

7. The culture of claim 6, wherein the microglia are at least 90% positive for TREM2, P2RY12, TMEM119, IBA-1, and/or CX3CR1.

8. The culture of claim 7, wherein the microglia are mature microglia.

9. The culture of any of claims 1-8, wherein the astrocytes are positive for S100 beta, GFAP, and CD44.

10. The culture of any of claims 1-9, wherein the neurons are positive for at least two of the markers selected from the group consisting of SCL1, BCL11B, Calb2, CD24, CDH1, CUX1 Cux2, DCX, DLG4, Dlx, Dlx2, Emx1, Emx2, eomes, ETV1, FOXG1, FOXP2, Fut4, GABRA2, GAD1, GAD2, GAPDH, GFAP, GRIN2B, HoxB4, HTR2C, ISL1, ITGB1, LHX2,Neurog1, NKX2-1, Nos1, NPY, NR4A2, PAX6,

POU3F2, PVALB, RELN , SATB2, SLC17A6, SLC17A7, SLC17A8, SLC32A1, SOX1, Sox10, SST, SYN1, and Tbr1.

11. The culture of any of claims 1-6, wherein the microglia are derived from donors expressing disease-associated SNPs.

12. The culture of any of claims 1-11, wherein the microglia are generated from disease associated iPSC donors with TREM2, APOE, CD33, BIN, ABCA7, SNPS or genotypes associated with neurodegeneration.

13. The culture of any of claims 1-11, wherein the microglia comprise a disruption in TREM2, Methyl-CpG Binding Protein 2 (MeCP2), and/or Alpha-synuclein (SCNA).

14. The culture of any of claims 1-13, wherein the microglia comprise a disruption in TREM2.

15. The culture of claim 14, wherein the disruption in TREM2 comprises a TAL nuclease-mediated disruption at amino acid 58 in exon 2 of TREM2.

16. The culture of claim 14, wherein the microglia comprise a heterozygous disruption of TREM2.

17. The culture of claim 14, wherein the microglia comprise a homozygous disruption of TREM2.

18. The culture of any of claims 1-16, wherein the cell culture is a two-dimensional (2D) culture.

19. The culture of any of claims 1-18, wherein the media further comprises IL-34 and M-CSF or analogs or mimetics thereof.

20. The culture of any of claims 1-19, wherein the media further comprises IL-34 at a concentration of 100 ng/mL and M-CSF at a concentration of 25 ng/mL.

21. The culture of any of claims 1-20, wherein the media further comprises TGFβ or analogs or mimetics thereof.

22. The culture of any of claims 1-21, wherein the media comprises TGFβ at a concentration of 50 ng/mL.

23. The culture of any of claims 1-22, wherein the cells are cultured on a cell surface.

24. The culture of any of claims 1-23, wherein the cells are cultured on a surface coated with polyethyleneimine (PEI).

25. The culture of any of claims 1-24, wherein the cells are cultured on a surface coated with an extracellular matrix protein.

26. The culture of claim 26, wherein the extracellular matrix is basement membrane extract (BME) purified from murine Engelbreth-Holm-Swarm tumor.

27. The culture of claim 25, wherein the extracellular matrix protein is MATRIGEL®, GELTREX™, collagen, or laminin.

28. The culture of claim 25 or 26, wherein the extracellular matrix protein is GELTREX™.

29. The culture of claim 25 or 26, wherein the extracellular matrix protein is laminin.

30. The culture of any of claims 1-28, wherein the cell culture is a three-dimensional (3D) culture.

31. The culture of claim 29, wherein the 3D culture is a brain organoid culture.

32. The culture of any of claims 30-31, wherein the 3D culture comprises a functional neuron network.

33. The culture of claims 32, wherein the functional neuron network comprises calcium oscillations.

34. The culture of any of claims 1-33, wherein the microglia, astrocytes and neurons are isogenic.

35. The culture of any of claims 1-34, wherein the cultures comprises the microglia and astrocytes in a ratio of 1:1.

36. The culture of any of claims 1-34, wherein the cultures comprises the microglia, astrocytes, and neurons in a ratio of 1:1:5.

37. The culture of any of claims 1-36, wherein the culture comprises microglia at a cell density of 15,000 cells/cm^2 to 25,000 cells/cm^2.

38. The culture of any of claims 1-36, wherein the culture comprises neurons at a cell density of 125,000 cells/cm^2 to 160,000 cells/cm^2.

39. The culture of any of claims 1-36, wherein the culture comprises astrocyte at a cell density of 25,000 cells/cm^2 to 35,000 cells/cm^2.

40. The culture of any of claims 1-36, wherein the microglia, astrocytes, and neurons have been in the culture for at least 14 days.

41. The culture of claim 40, wherein the microglia, astrocyte, and neurons are present at a ratio of 2:6:1.

42. The culture of any of claims 1-40, wherein the iPSCs are human.

43. The culture of any of claims 1-42, wherein the culture is xeno-free, feeder-free, and/or conditioned-media free.

44. The culture of any of claims 1-42, wherein the media is defined media.

45. A cell culture comprising brain microvascular endothelial cells (BMECs), pericytes, and astrocytes in a sandwich format.

46. The culture of claim 45, further defined as a blood brain barrier model.

47. The culture of claim 45 or 46, wherein the sandwich format comprises an extracellular matrix layer between two cell layers.

48. The culture of claim 47, wherein the extracellular matrix layer comprises at least two extracellular matrix proteins.

49. The culture of claim 48, wherein the at least two extracellular matrix proteins are collagen IV and fibronectin.

50. The culture of any of claims 45-48, wherein the sandwich format comprises BMECs on the apical side, the extracellular matrix layer in the middle, and astrocytes and pericytes on the basolateral side.

51. The culture of claim 50, wherein the extracellular matrix layer further comprises gelatin and the astrocytes and pericytes.

52. The culture of any of claims 45-50, wherein the sandwich format further comprises a permeable membrane insert.

53. The culture of claim 52, wherein the permeable membrane insert is a polytetrafluoroethylene (PFTE), polycarbonate, or polyethylene terephthalate (PTE) insert.

54. The culture of claim 52, wherein the permeable membrane insert is a PFTE insert.

55. The culture of claim 54, wherein the PFTE insert is a TRANSWELL™ insert.

56. The culture of any of claims 45-55, wherein the permeable membrane insert is coated with human collagen IV and human fibronectin on the apical side.

57. The culture of claim 56, wherein the permeable membrane insert is coated with human collagen IV at a concentration of 200-400 µg/mL and human fibronectin at a concentration of 50-100 µg/mL.

58. The culture of any of claims 45-57, wherein the basolateral side of the permeable membrane insert is coated with gelatin.

59. The culture of claims 58, wherein the basolateral side of the permeable membrane insert is coated with 0.1% gelatin.

60. The culture of any of claims 45-59, wherein the astrocytes and pericytes are present at a 1:2 ratio.

61. The culture of any of claims 45-60, wherein the astrocytes and pericytes are on the basolateral side of the permeable membrane insert.

62. The culture of any of claims 45-61, wherein the BMECs are on the apical side of the permeable membrane insert.

63. The culture of any of claims 45-62, wherein the BMECs are in media comprising EFRA2.

64. The culture of any of claims 45-63, wherein the BMECs, astrocytes and pericytes are in media comprising a ROCK inhibitor.

65. The culture of claim 64, wherein the ROCK inhibitor is Y-27632.

66. The culture of any of claims 45-65, wherein the BMECs, astrocytes, and pericytes are present at a ratio of 4:1:2, 4:1:1, 4:2:1, or 2:1:1.

67. The culture of any of claims 45-65, wherein the BMECs are seeded at a cell density of 1 x10^6 cells/cm^2 to 1.5x10^6 cells/cm^2. 1 (1.3x106)

68. The culture of any of claims 45-65, wherein the astrocytes are seeded at a cell density of 300,000 cells/cm^2 to 700,000 cells/cm^2. (333K)

69. The culture of any of claims 45-65, wherein the pericytes are seeded at a cell density of 300,000 cells/cm^2 to 700,000 cells/cm^2. (666K)

70. A method for screening a therapeutic compound for treating a neurodegenerative disease comprising:

(a) contacting a test compound with a culture of any of claims 1-66; and

(b) measuring the functional activity of the cells.

71. The method of claim 70, wherein an increase in functional activity indicates the test compound is capable of treating a neurodegenerative disease.

72. The method of claim 70, wherein measuring functional activity comprises measuring dendrite area (MAP2), synapse count, cell count, or axon area.

73. The method of claim 70, wherein measuring the functional activity comprises detecting release of complement C3 from microglia.

74. The method of claim 73, wherein a decrease in complement C3 released from the microglia indicates that the therapeutic compound is capable of treating a neurodegenerative disease.

75. The method of claim 70, further comprising contacting the culture with LPS.

76. The method of claim 75, wherein measuring functional activity comprises measuring analytes released in the media with and without stimulation with LPS.

77. The method of claim 76, wherein the analytes are M1 factors.

78. The method of claim 77, wherein the M1 factors are TNF alpha, IL-6, CCL2, CCL3, CCL4, IL-1 beta, IL-12, IL-13, IL-8, Interferon gamma, IL1-Alpham FAS ligand, IL-2, GMCSF, Granzyme B, ICAM-1, and/or CXCL11.

79. The method of claim 76, wherein the analytes are M2 factors.

80. The method of claim 79, wherein the M2 factors are IL-4, IL-10, IL-21, VEGF, CCL5, IL-17, IL1-RII, GCSG, CXCL5.

81. The method of any of claims 70-80, wherein step (a) comprises a culture with TREM2 wild-type microglia, a culture with TREM2 heterozygous knockout microglia, and/or a culture with TREM2 homozygous knockout microglia.

82. The method of claim 70, wherein measuring functional activity comprises measuring neural function by calcium signaling or microelectrode array (MEA).

83. The method of claim 70, wherein measuring functional activity comprises measuring amyloid beta phagocytic function.

84. The method of claim 70, wherein the neurodegenerative disease is Alzheimer's disease or multiple sclerosis.

85. Use of the culture of any of claims 1-66 as a model of a neurodegenerative disease.

86. The use of claim 85, wherein the model comprises a culture with TREM2 wild-type microglia, a culture with TREM2 heterozygous knockout microglia, and/or a culture with TREM2 homozygous knockout microglia.

87. The use of claim 85, wherein the model comprises engineered or patient specific iPSC derived astrocytes, neurons and/or microglia harboring disease relevant genotypes SNPs or mutations in APOE4/4, CD33, ABCA, BIN1, or R47H.

88. A method of screening for a neurodegenerative disease comprising detecting a level of soluble TREM2 in a culture of any of claims 1-66.

89. The method of claim 88, wherein cells in the culture are derived from isogenically engineered iPSC lines or from donors expressing disease associated SNPs or mutations associated with neurodegeneration.

90. The method of claim 88 or 89, wherein the level of soluble TREM2 is detected in conditioned media.

91. The method of claim 90, wherein detecting comprises performing an ELISA.

92. The method of any of claims 88-91, wherein detecting an increased level of soluble TREM2 as compared to a control indicates the presence of a neurodegenerative disease.

93. The method of any of claims 88-92, further comprising detecting the level of COMT, NRXN2 and/or SST in microglia.

94. The method of any of claims 88-92, wherein the neurodegenerative disease is Alzheimer's disease or multiple sclerosis.

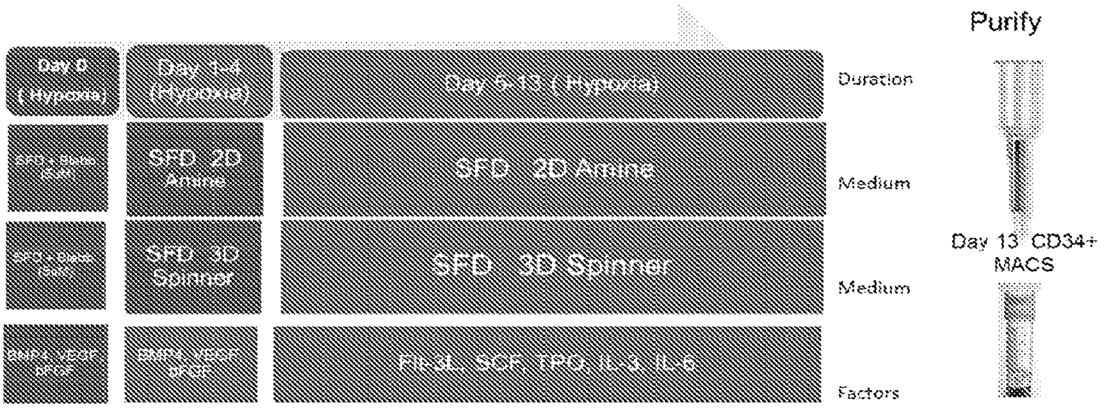

Purify
Day 0 (Hypoxia)
Day 1-4 (Hypoxia)
Day 5-13 (Hypoxia)
Duration
SFD + Blebb
SFD 2D Amine
SFD 2D Amine
Medium
SFD + Blebb
SFD 3D Spinner
SFD 3D Spinner
Medium
Day 13 CD34+ MACS
BMP4, VEGF bFGF
BMP4, VEGF bFGF
Flt-3L, SCF, TPO, IL-3, IL-6
Factors

FIG. 1

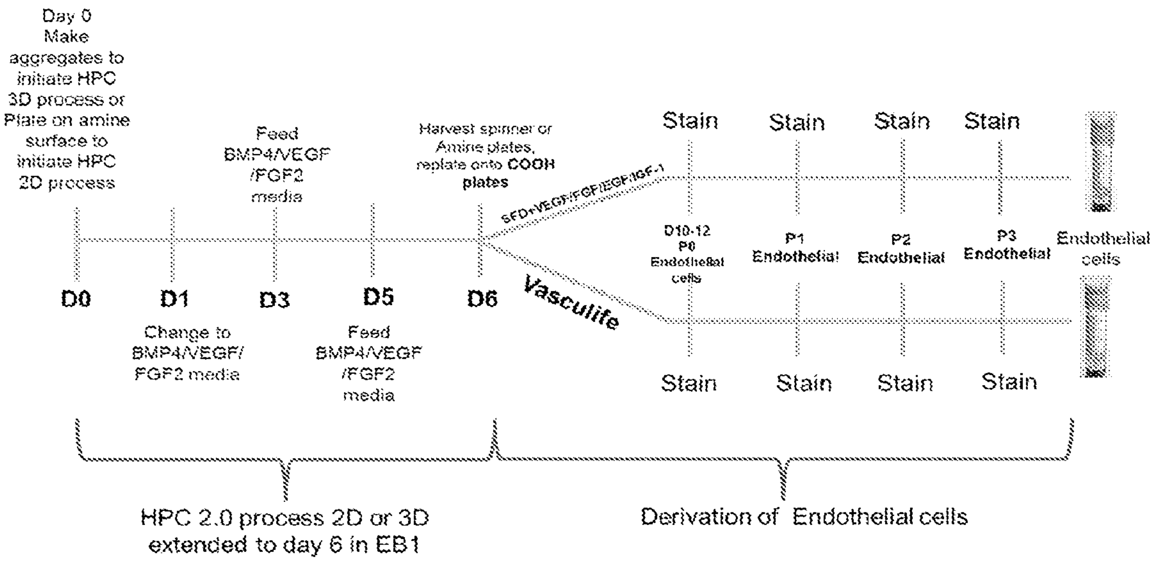

Day 0
Make aggregates to initiate HPC 3D process or Plate on amine surface to initiate HPC 2D process
Feed BMP4/VEGF /FGF2 media
Harvest spinner or Amine plates, replate onto COOH plates
Stain
Stain
Stain
Stain
SFD+VEGF/FGF/EGF/IGF-1
D10-12 P0 Endothelial cells
P1 Endothelial
P2 Endothelial
P3 Endothelial
Endothelial cells
D0
D1
D3
D5
D6
Vasculife
Change to BMP4/VEGF/ FGF2 media
Feed BMP4/VEGF /FGF2 media
Stain
Stain
Stain
Stain
HPC 2.0 process 2D or 3D extended to day 6 in EB1
Derivation of Endothelial cells

FIG. 2

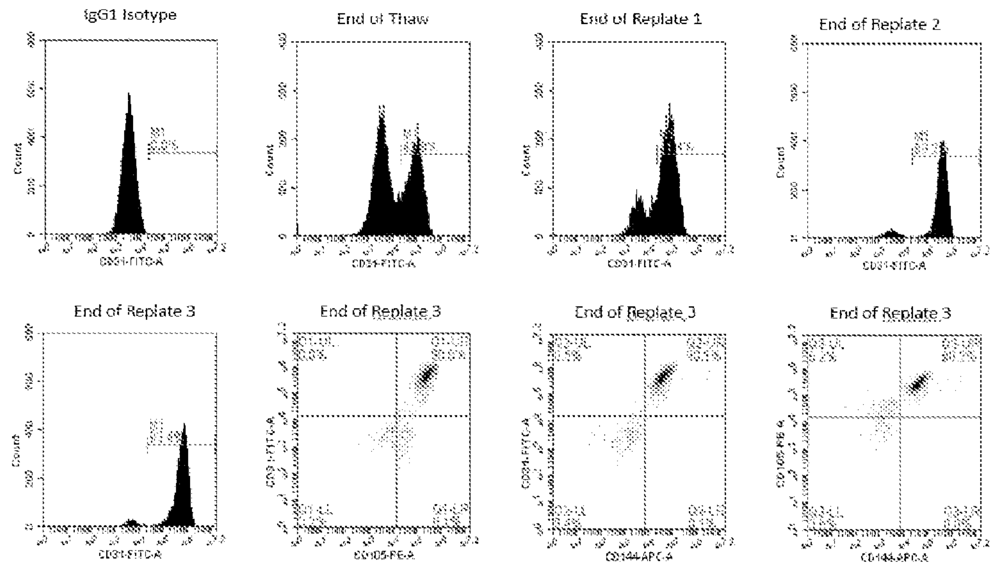

IgG1 Isotype
End of Thaw
End of Replate 1
End of Replate 2
End of Replate 3
End of Replate 3
End of Replate 3
End of Replate 3
Count
CD31-FITC-A
CD105-PE-A
CD144-APC-A

FIG. 3

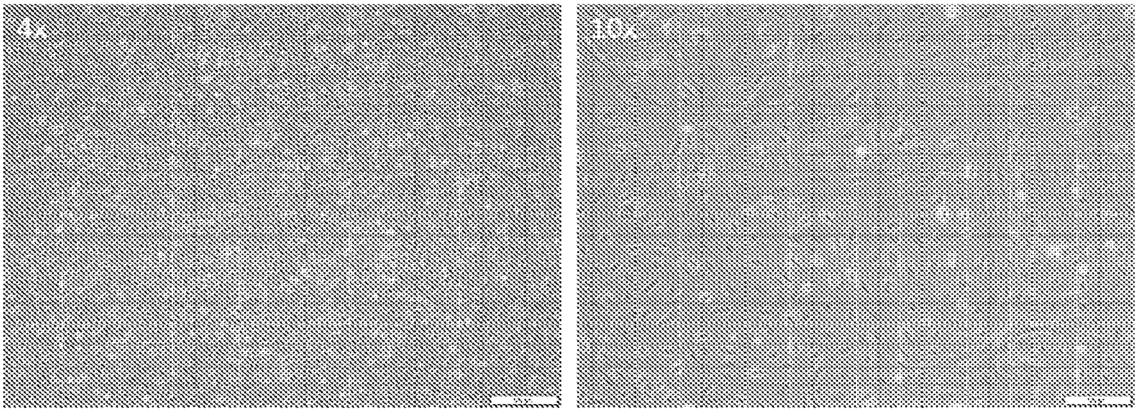


FIG. 4A

SFD	
IMDM	75%
Ham's F12	25%
N2 (CTS)	0.5%
B27 w/o RA (CTS)	1%
HSA	0.05%
MTG	450uM
Ascorbic Acid	50ug/mL
Pen/Strep	1%
GlutaMAX	1%

SFD EB#1 Medium	
SFD	100%
BMP4	50ng/mL
FGF-b	50ng/mL
VEGF	50ng/mL

SFD Endothelial Medium	
SFD	100%
FGF-b	25ng/mL
EGF	5ng/mL
VEGF	5ng/mL
IGF-1	30ng/mL
Heparin	0.75U/mL
Hydro-cortisone	1ug/mL

VascuLife VEGF Endo	
Vasc Basal	90%
FBS	10%
FGF-b	5ng/mL
VEGF	5ng/mL
EGF	5ng/mL
Heparin Sulfate	0.75U/mL
L-Glutamine	10mM
IGF-1	15ng/mL
Ascorbic Acid	50ug/mL
Hydro-cortisone	1ug/mL

FIG. 4B

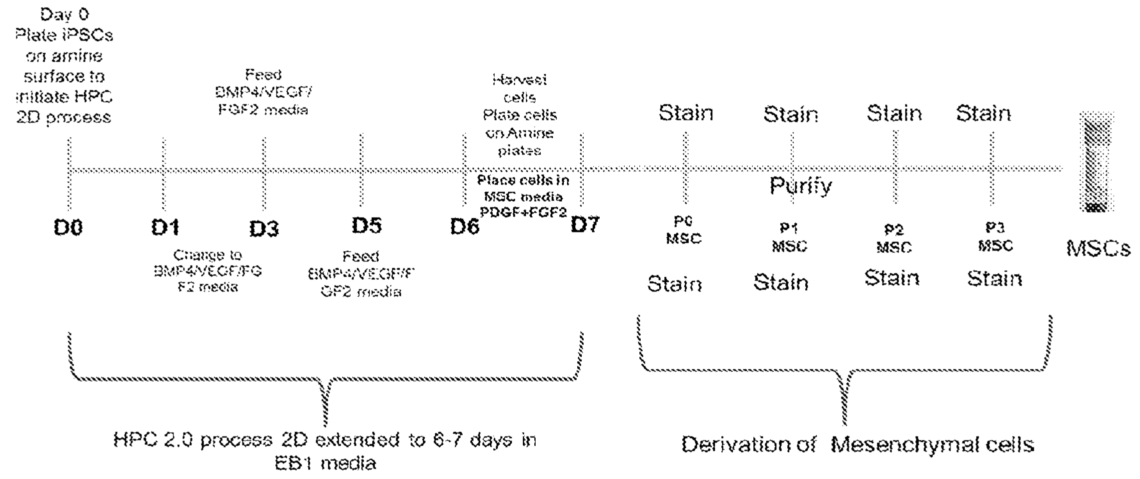

Day 0
Plate iPSCs on amine surface to initiate HPC 2D process
Feed BMP4/VEGF/FGF2 media
Harvest cells Plate cells on Amine plates
Place cells in MSC media PDGF+FGF2
Stain
Stain
Stain
Stain
Purify
D0
D1
D3
D5
D6
D7
P0 MSC
P1 MSC
P2 MSC
P3 MSC
MSCs
Change to BMP4/VEGF/FGF2 media
Feed BMP4/VEGF/FGF2 media
Stain
Stain
Stain
Stain
HPC 2.0 process 2D extended to 6-7 days in EB1 media
Derivation of Mesenchymal cells

FIG. 5A

"GMP" SFD	
IMDM	75%
Ham's F12	25%
N2 (CTS)	0.5%
B27 w/o RA (CTS)	1%
HSA	0.05%
MTG	450uM
Ascorbic Acid	50ug/mL
Pen/Strep	1%
GlutaMAX	1%

"GMP" EB#1	
"GMP" SFD	
GMP BMP4	50ng/mL
GMP VEGF	50ng/mL
GMP FGF2	50ng/mL

"GMP" MSC Maintanenice Medium (SFD)	
"GMP" SFD	
GMP PDGF-BB	50ng/mL
GMP FGF2	50ng/mL

FIG. 5B

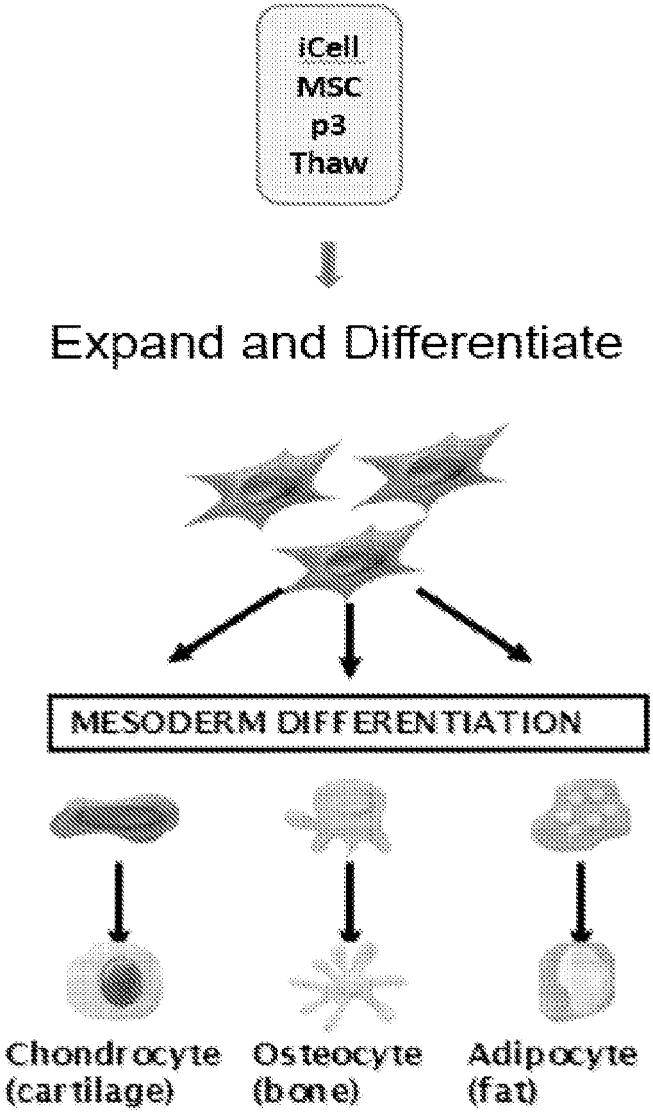

iCell
MSC
p3
Thaw
Expand and Differentiate
MESODERM DIFFERENTIATION
Chondrocyte
(cartilage)
Osteocyte
(bone)
Adipocyte
(fat)

FIG. 5C

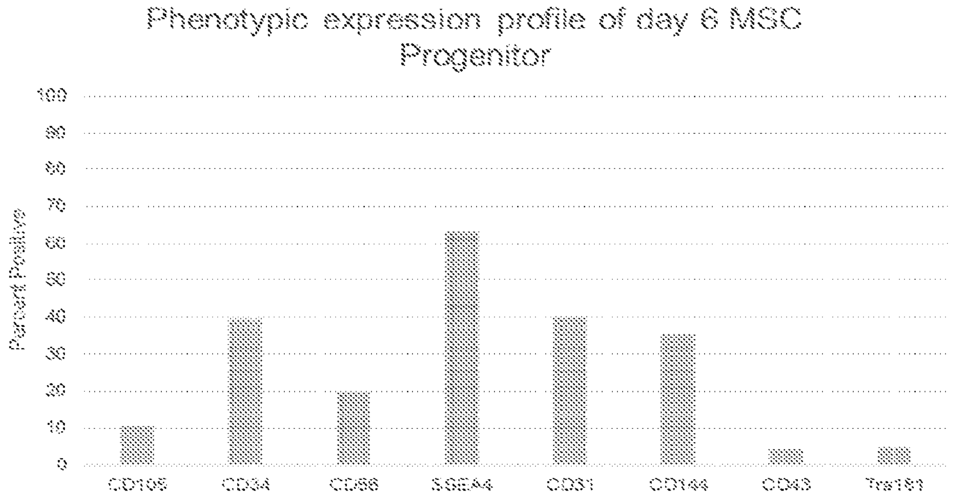

Phenotypic expression profile of day 6 MSC Progenitor
Percent Positive
100
90
80
70
60
50
40
30
20
10
0
CD105
CD34
CD56
SSEA4
CD31
CD144
CD43
Tra181

FIG. 5D

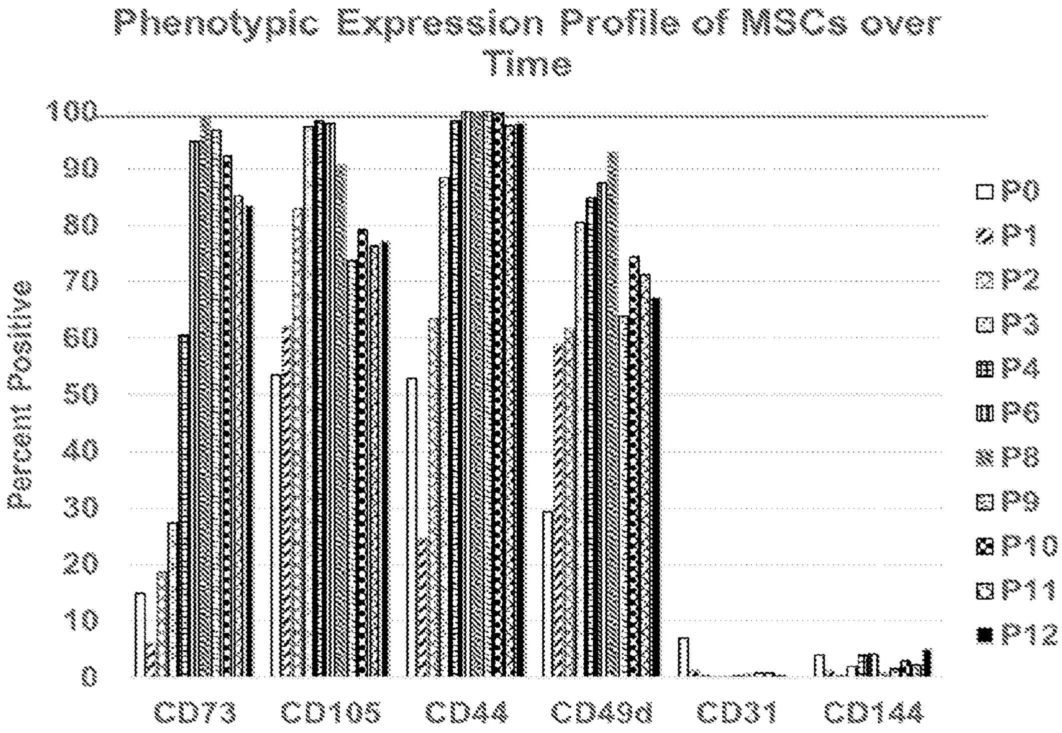

Phenotypic Expression Profile of MSCs over Time
Percent Positive
100
90
80
70
60
50
40
30
20
10
0
CD73
CD105
CD44
CD49d
CD31
CD144
P0
P1
P2
P3
P4
P6
P8
P9
P10
P11
P12

FIG. 6

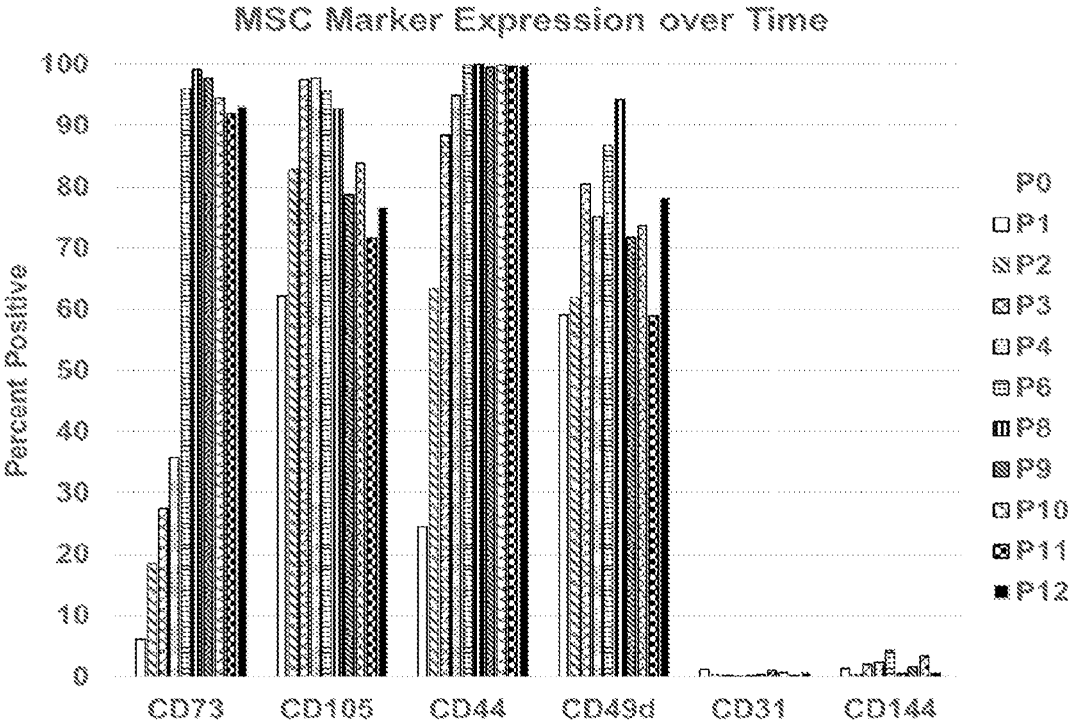

MSC Marker Expression over Time
Percent Positive
100
90
80
70
60
50
40
30
20
10
0
CD73
CD105
CD44
CD49d
CD31
CD144
P0
P1
P2
P3
P4
P6
P8
P9
P10
P11
P12

FIG. 7

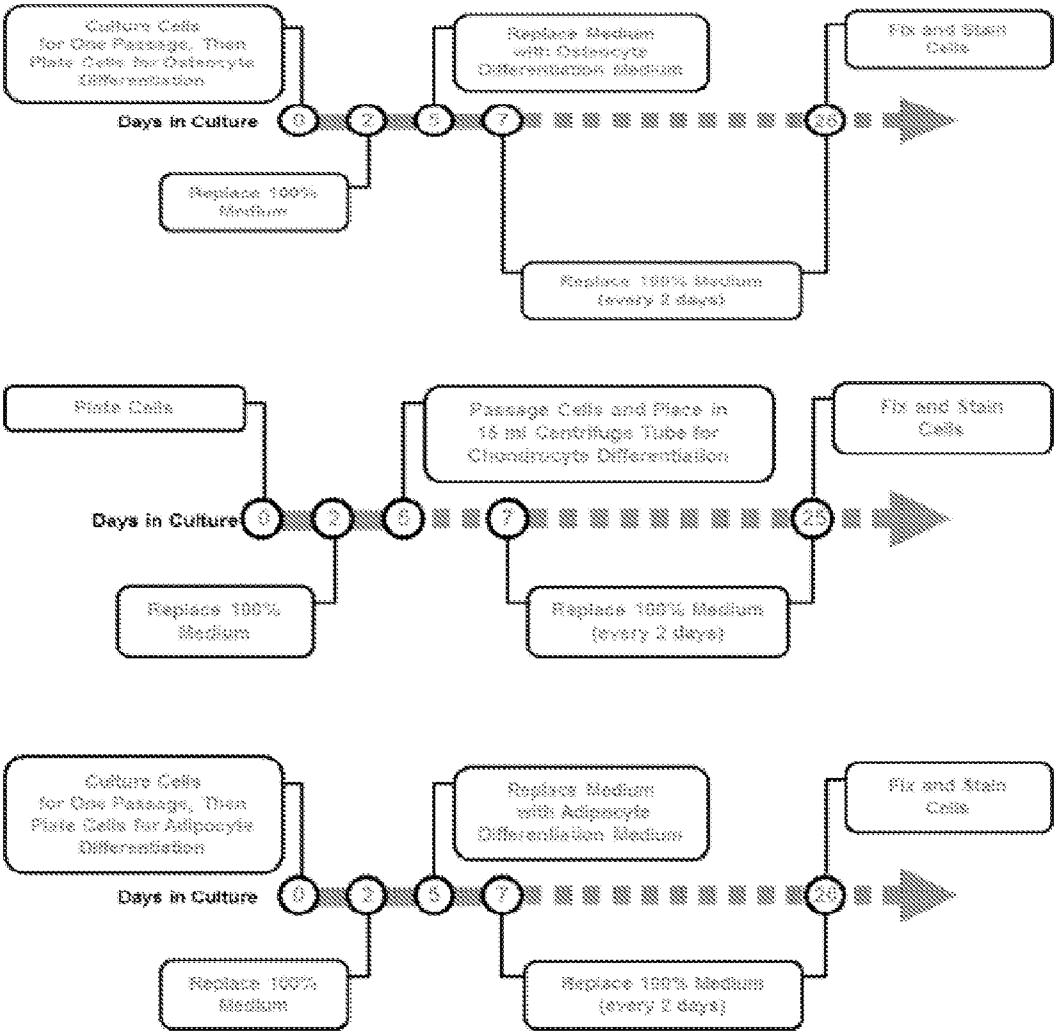

Culture Cells for One Passage, Then Plate Cells for Osteocyte Differentiation
Replace Medium with Osteocyte Differentiation Medium
Fix and Stain Cells
Days in Culture
0
2
5
7
28
Replace 100% Medium
Replace 100% Medium (every 2 days)
Plate Cells
Passage Cells and Place in 15 ml Centrifuge Tube for Chondrocyte Differentiation
Fix and Stain Cells
Days in Culture
0
2
5
7
28
Replace 100% Medium
Replace 100% Medium (every 2 days)
Culture Cells for One Passage, Then Plate Cells for Adipocyte Differentiation
Replace Medium with Adipocyte Differentiation Medium
Fix and Stain Cells
Days in Culture
0
2
5
7
28
Replace 100% Medium
Replace 100% Medium (every 2 days)

FIG. 8A

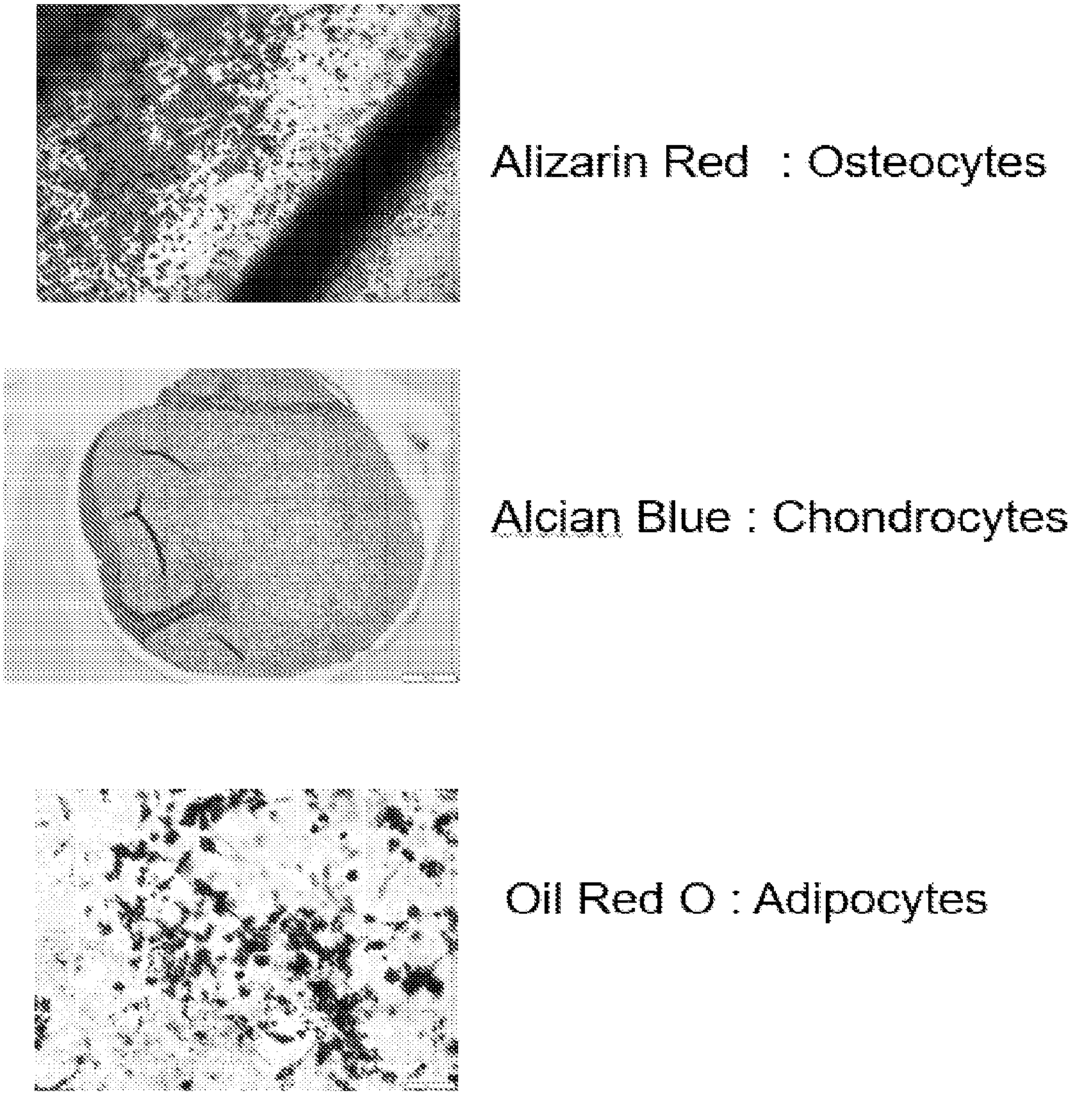

Alizarin Red : Osteocytes
Alcian Blue : Chondrocytes
Oil Red O : Adipocytes

FIG. 8B

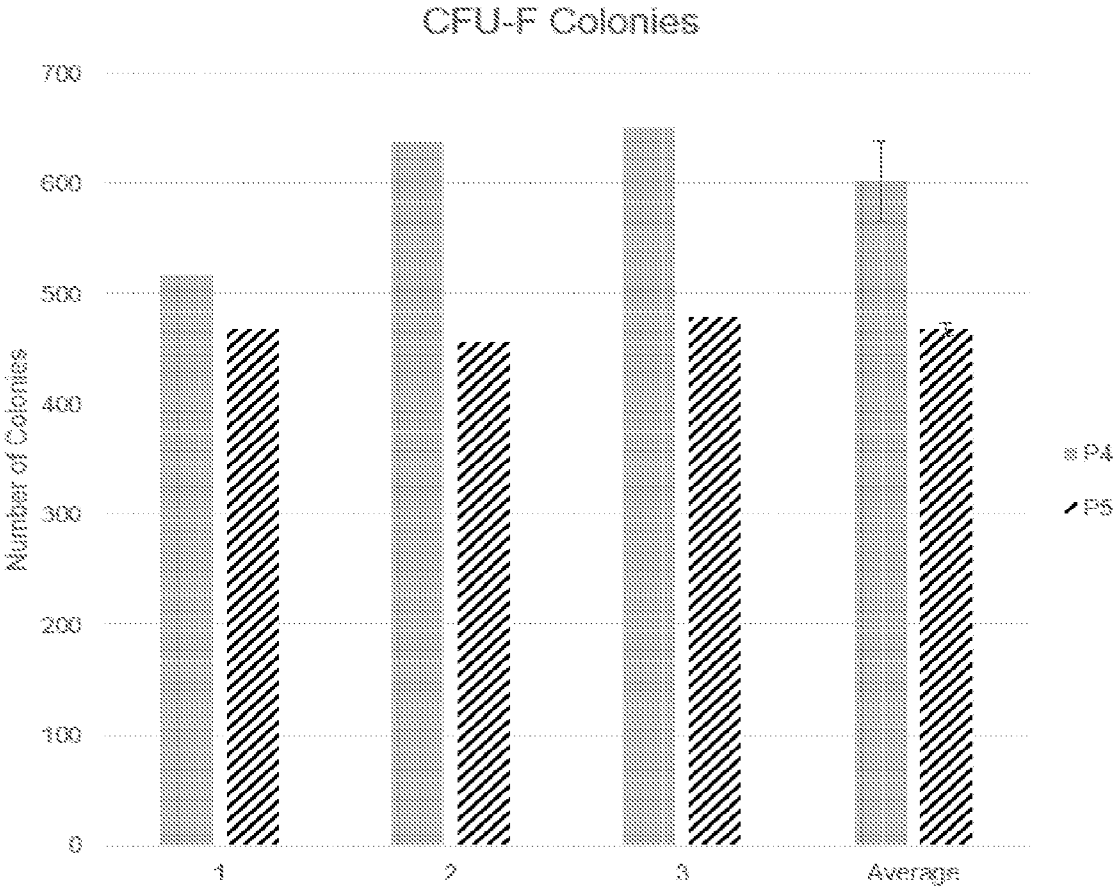

CFU-F Colonies
700
600
500
400
300
200
100
0
Number of Colonies
1
2
3
Average
P4
P5

FIG. 8C

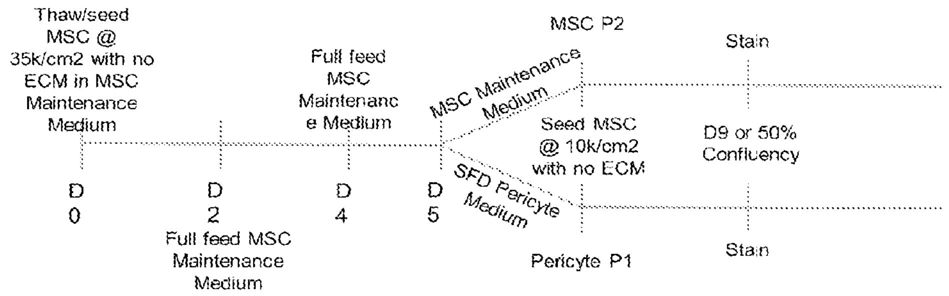

Thaw/seed MSC @ 35k/cm2 with no ECM in MSC Maintenance Medium
Full feed MSC Maintenance Medium
Full feed MSC Maintenance Medium
MSC P2
Stain
MSC Maintenance Medium
Seed MSC @ 10k/cm2 with no ECM
D9 or 50% Confluency
SFD Pericyte Medium
D 0
D 2
D 4
D 5
Pericyte P1
Stain

FIG. 9A

SFD Pericyte Medium	
SFD	100%
FGF-b	50ng/mL
EGF	25ng/mL
IGF-1	25ng/mL
Hydro-cortisone	1ug/mL

FIG. 9B

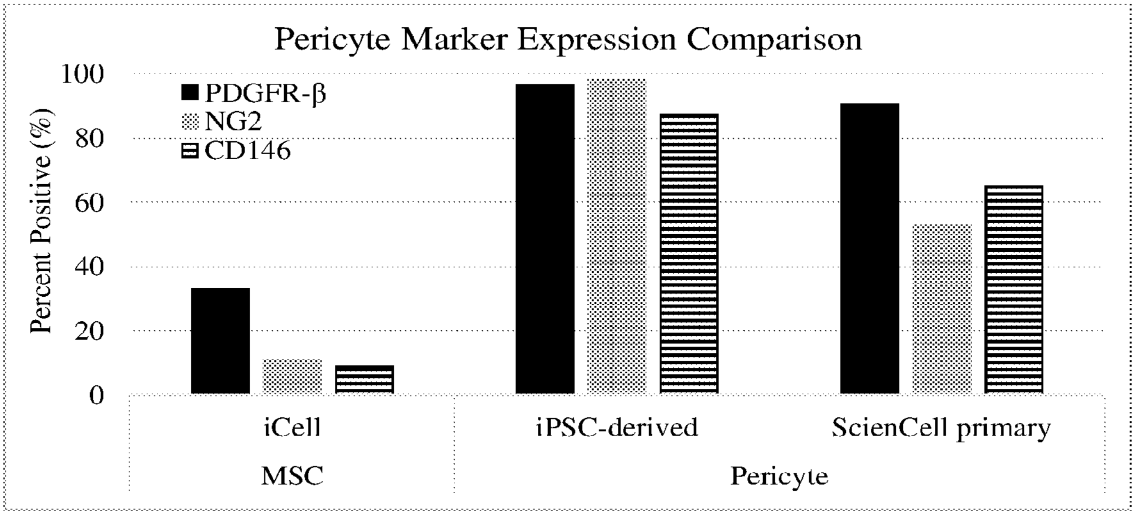


FIG. 9C

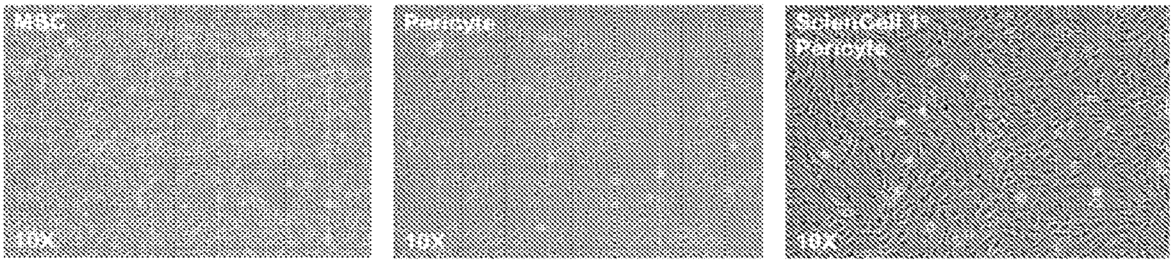


FIG. 9D

Subtype	Phenotype	PDGFR-β	NG2	CD13	CD146	CD274	VCAM1	Calponin	Desmin	DLK1	αSMA
PC1	Capillary / pro-inflammatory	+	+	+	+	+	+	low/-	low/-	-	-
PC2	Arteriolar / contractile	+	+	+	+	-	-	-	+	+	+

FIG. 9E

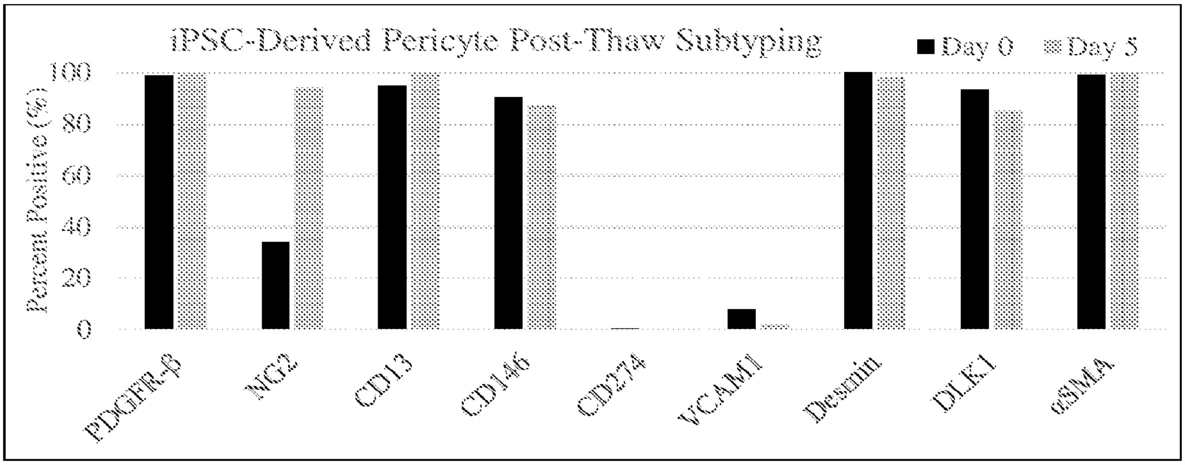

iPSC-Derived Pericyte Post-Thaw Subtyping
■ Day 0
▨ Day 5
Percent Positive (%)
100
80
60
40
20
0
PDGFR-β
NG2
CD13
CD146
CD274
VCAM1
Desmin
DLK1
αSMA

FIG. 9F

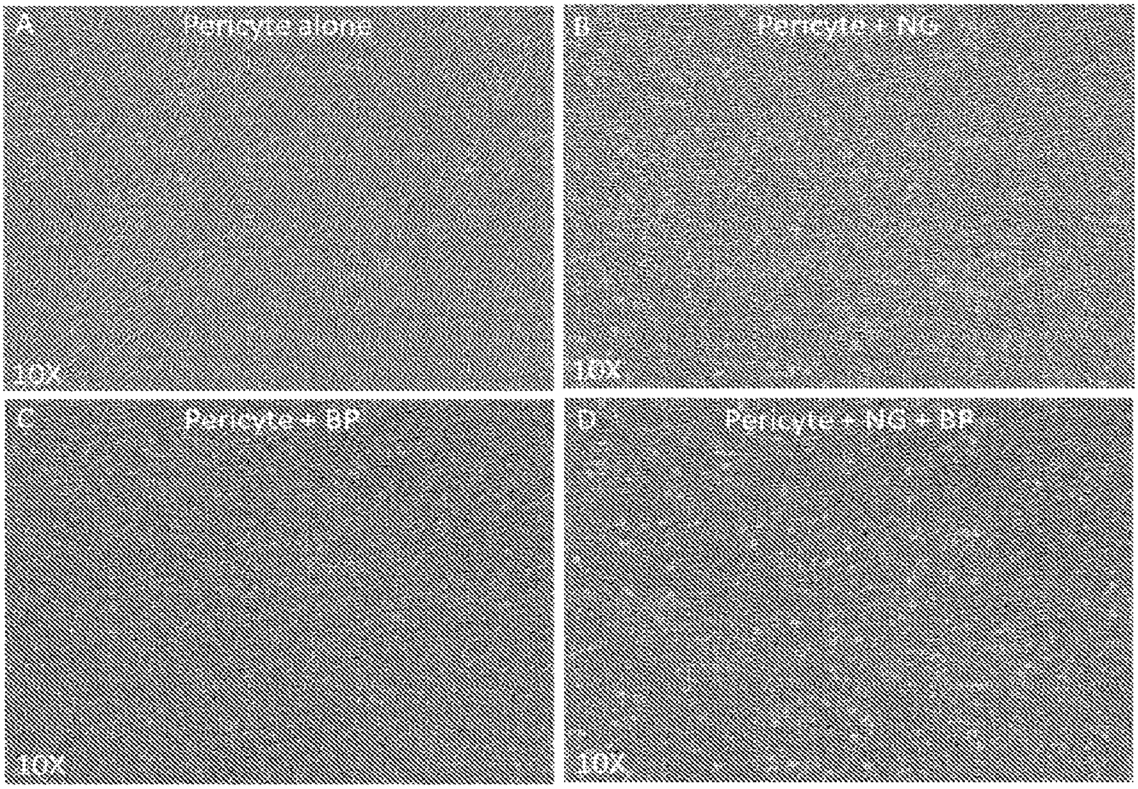

A
Pericyte alone
10X
B
Pericyte + NG
10X
C
Pericyte + BP
10X
D
Pericyte + NG + BP
10X

FIG. 9G

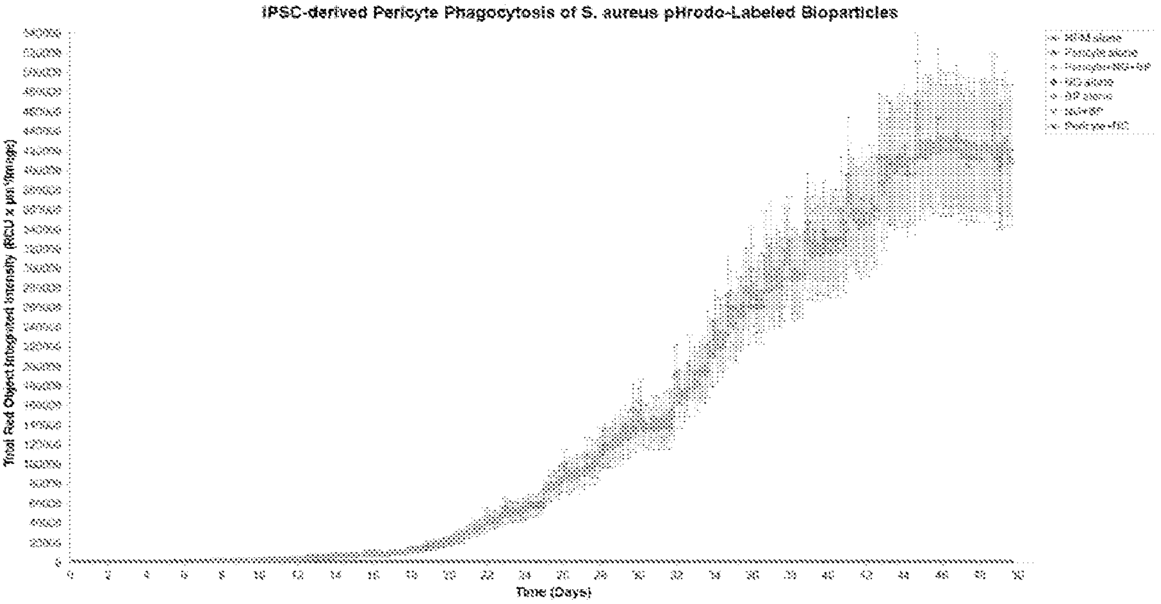


FIG. 9H

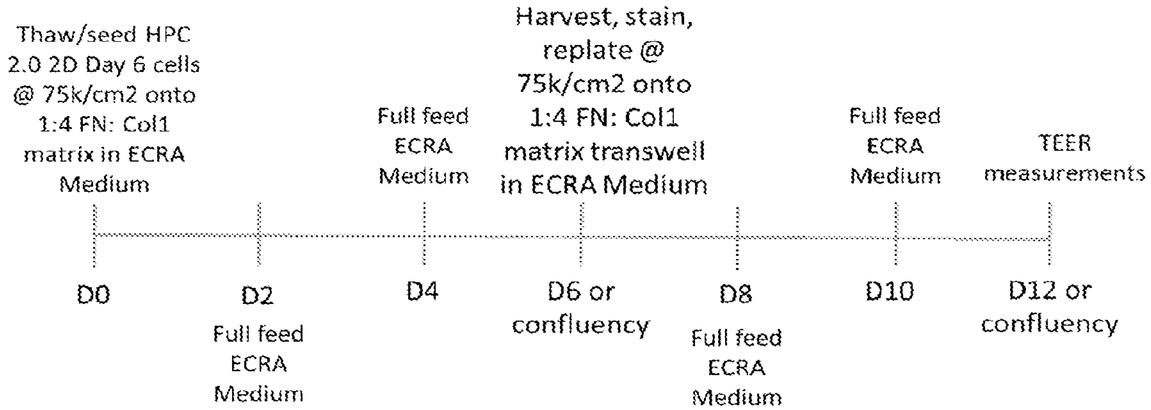

Thaw/seed HPC 2.0 2D Day 6 cells @ 75k/cm2 onto 1:4 FN: Col1 matrix in ECRA Medium
Full feed ECRA Medium
Harvest, stain, replate @ 75k/cm2 onto 1:4 FN: Col1 matrix transwell in ECRA Medium
Full feed ECRA Medium
TEER measurements
D0
D2
Full feed ECRA Medium
D4
D6 or confluency
D8
Full feed ECRA Medium
D10
D12 or confluency

FIG. 10A

ECRA Medium	
Human Endothelial SFM	100%
1% Platelet-poor plasma-derived bovine serum	20ng/mL
FGF-b	20ng/mL
Retinoic Acid	10uM

FIG. 10B

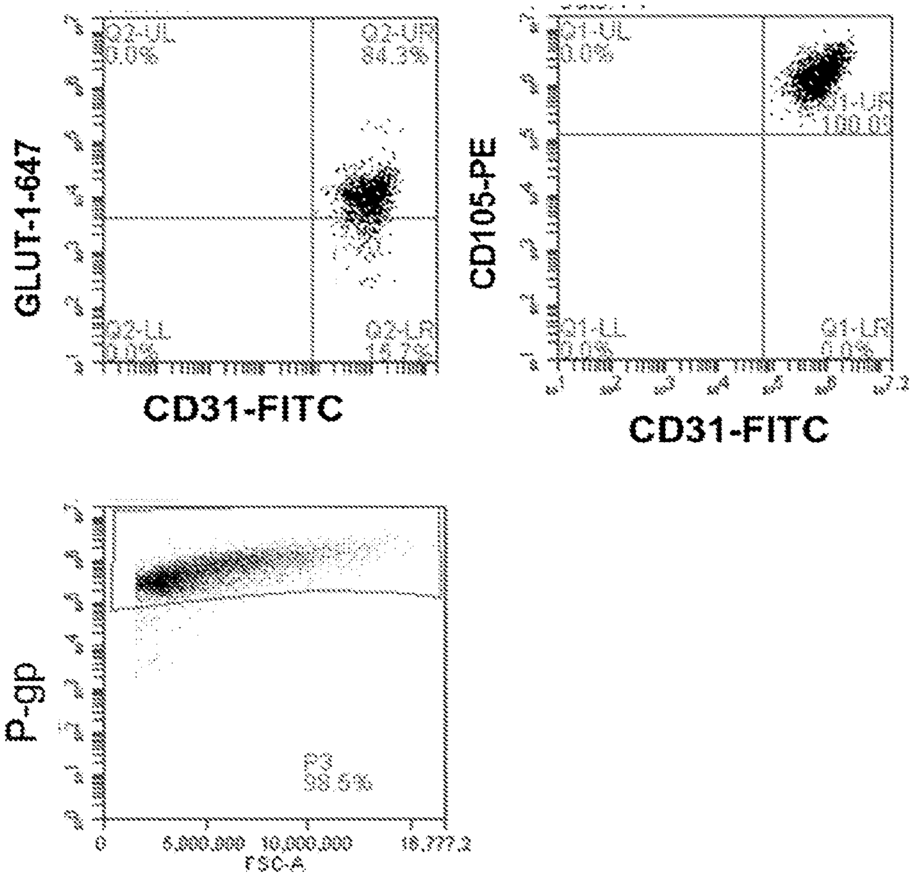
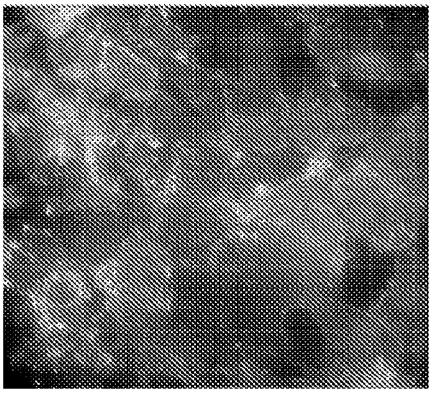


FIG. 10C



P-gp (Green), Nuclei (Blue)

FIG. 10D

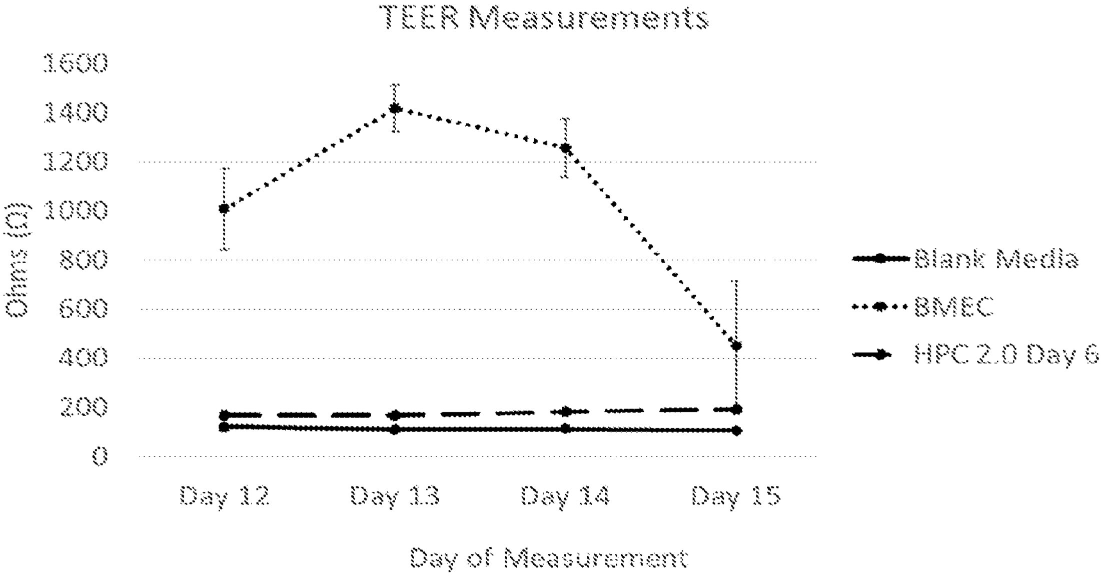
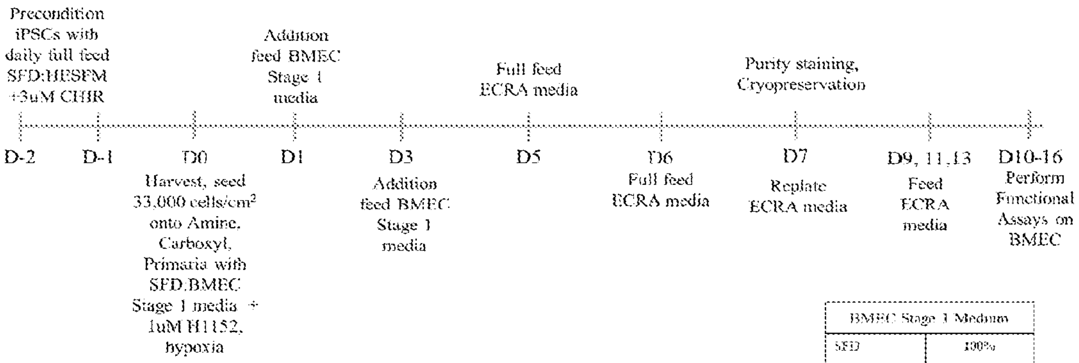


FIG. 10E


Precondition iPSCs with daily full feed SFD:HESFM +3uM CHIR
Addition feed BMEC Stage 1 media
Full feed ECRA media
Purity staining, Cryopreservation
D-2
D-1
D0
Harvest, seed 33,000 cells/cm^2 onto Amine, Carboxyl, Primaria with SFD:BMEC Stage 1 media + 1uM H1152, hypoxia
D1
D3
Addition feed BMEC Stage 1 media
D5
D6
Full feed ECRA media
D7
Replate ECRA media
D9, 11,13
Feed ECRA media
D10-16
Perform Functional Assays on BMEC

BMEC Stage 1 Medium	
SFD	100%
BMP4	25ng/mL
VEGF	25ng/mL
FGF	50ng/mL
PDGF-AA	10ng/mL
EGF	10ng/mL
Thymosin-β4	100ng/mL

FIG. 10F

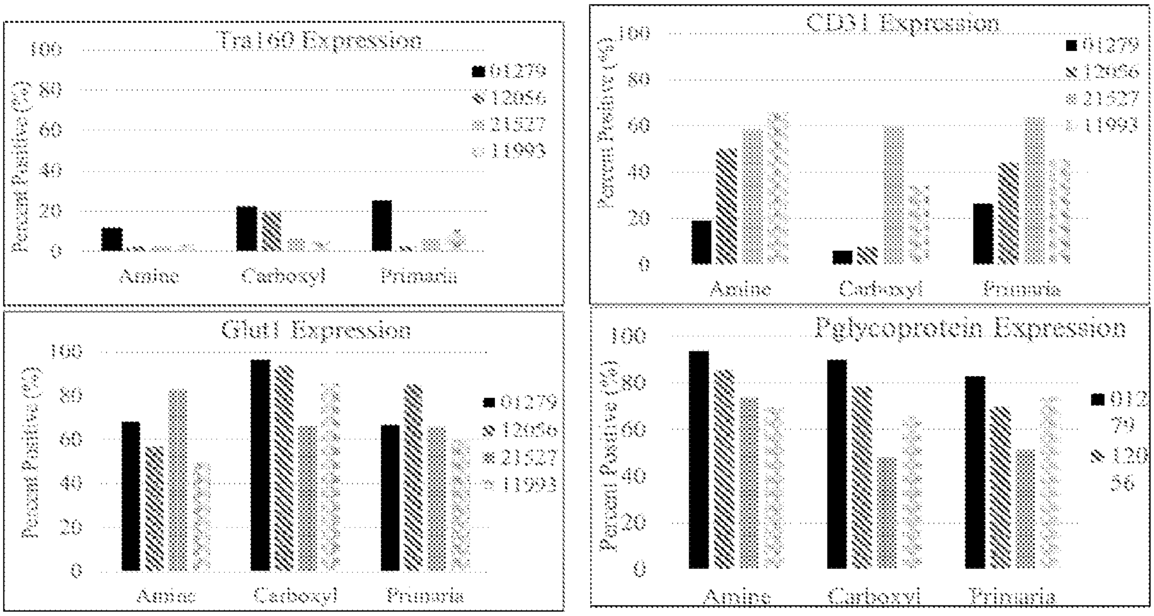
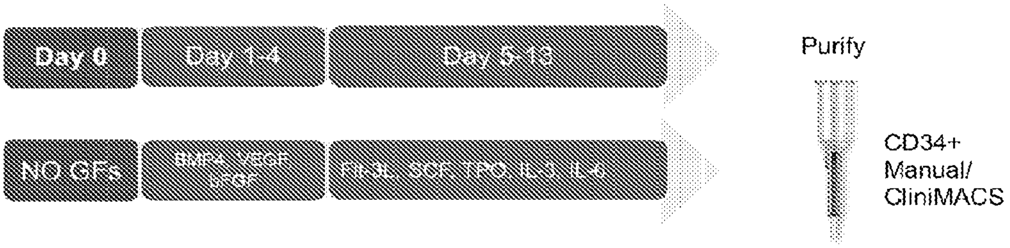


FIG. 10G



Starting iPSC cell number	Ending Cell number of unsorted HPCs	Actual Purified HPCs	CD34 Separation
7.20E+08	1.53E+09	2.81E+08	Manual MACS
4.93E+08	1.36E+09	2.10E+08	Manual MACS
1.45E+09	5.40E+09	4.83E+08	Manual MACS
8.15E+08	6.70E+09	4.95E+08	CliniMACS
1.09E+09	3.73E+09	2.83E+08	CliniMACS
1.50E+09	3.60E+09	3.51E+08	CliniMACS

FIG. 11

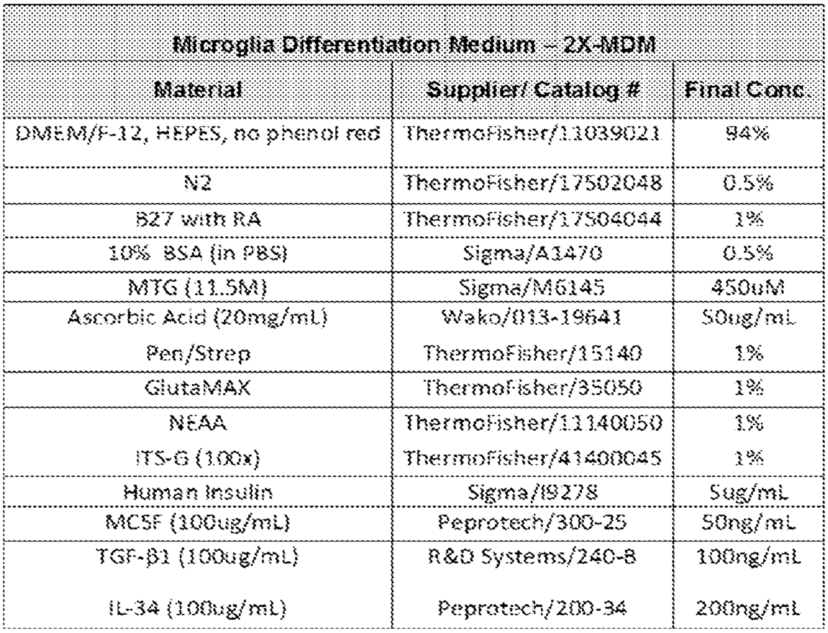
Microglia Differentiation Base Medium

Material	Supplier/ Catalog #	Final Conc.
DMEM/F-12, HEPES, no phenol red	ThermoFisher/11039021	94%
N2	ThermoFisher/17502048	0.5%
B27 with RA	ThermoFisher/17504044	1%
10% BSA (in PBS)	Sigma/A1470	0.05%
MTG (11.5M)	Sigma/M6145	450uM
Ascorbic Acid (20mg/mL)	Wako/013-19641	50ug/mL
Pen/Strep	ThermoFisher/15140	1%
GlutaMAX	ThermoFisher/35050	1%
NEAA	ThermoFisher/11140050	1%
ITS-G (100x)	ThermoFisher/41400045	1%
Human Insulin	Sigma/I9278	5ug/mL

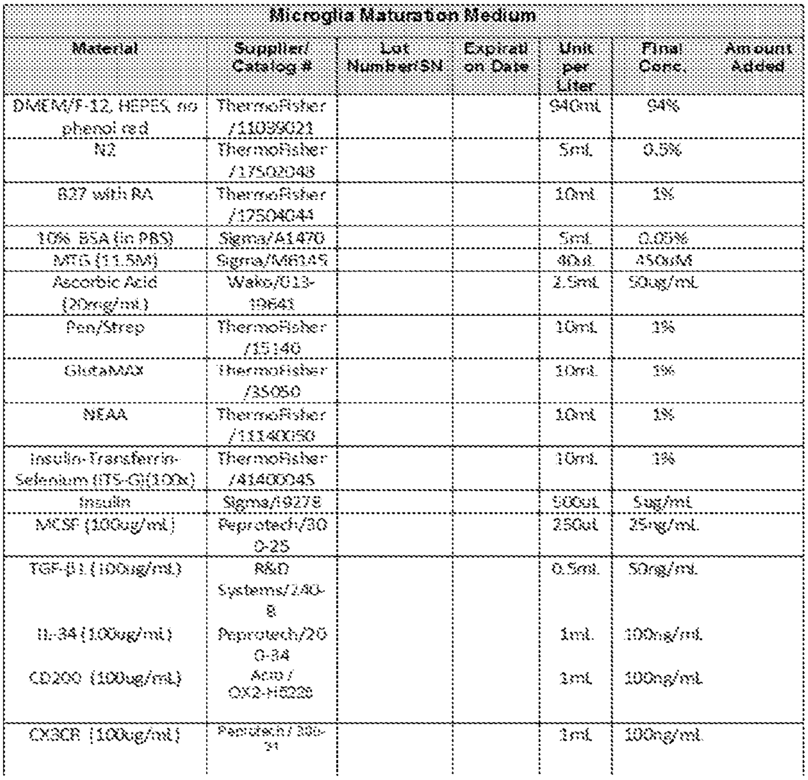
Microglia Differentiation Medium - MDM

Material	Supplier/ Catalog #	Final Conc.
DMEM/F-12, HEPES, no phenol red	ThermoFisher/11039021	94%
N2	ThermoFisher/17502048	0.5%
B27 with RA	ThermoFisher/17504044	1%
10% BSA (in PBS)	Sigma/A1470	0.5%
MTG (11.5M)	Sigma/M6145	450uM
Ascorbic Acid (20mg/mL)	Wako/013-19641	50ug/mL
Pen/Strep	ThermoFisher/15140	1%
GlutaMAX	ThermoFisher/35050	1%
NEAA	ThermoFisher/11140050	1%
ITS-G (100x)	ThermoFisher/41400045	1%
Human Insulin	Sigma/I9278	5ug/mL
MCSF (100ug/mL)	Peprotech/300-25	25ng/mL
TGF-β1 (100ug/mL)	R&D Systems/240-B	50ng/mL
IL-34 (100ug/mL)	Peprotech/200-34	100ng/mL

FIG. 12



Microglia Differentiation Medium – 2X-MDM		
Material	**Supplier/ Catalog #**	**Final Conc.**
DMEM/F-12, HEPES, no phenol red	ThermoFisher/11039021	94%
N2	ThermoFisher/17502048	0.5%
B27 with RA	ThermoFisher/17504044	1%
10% BSA (in PBS)	Sigma/A1470	0.5%
MTG (11.5M)	Sigma/M6145	450uM
Ascorbic Acid (20mg/mL)	Wako/013-19641	50ug/mL
Pen/Strep	ThermoFisher/15140	1%
GlutaMAX	ThermoFisher/35050	1%
NEAA	ThermoFisher/11140050	1%
ITS-G (100x)	ThermoFisher/41400045	1%
Human Insulin	Sigma/I9278	5ug/mL
MCSF (100ug/mL)	Peprotech/300-25	50ng/mL
TGF-β1 (100ug/mL)	R&D Systems/240-B	100ng/mL
IL-34 (100ug/mL)	Peprotech/200-34	200ng/mL



Microglia Maturation Medium						
Material	**Supplier/ Catalog #**	**Lot Number/SN**	**Expiration Date**	**Unit per Liter**	**Final Conc.**	**Amount Added**
DMEM/F-12, HEPES, no phenol red	ThermoFisher /11039021			940mL	94%	
N2	ThermoFisher /17502048			5mL	0.5%	
B27 with RA	ThermoFisher /17504044			10mL	1%	
10% BSA (in PBS)	Sigma/A1470			5mL	0.05%	
MTG (11.5M)	Sigma/M6145			40uL	450uM	
Ascorbic Acid (20mg/mL)	Wako/013-19641			2.5mL	50ug/mL	
Pen/Strep	ThermoFisher /15140			10mL	1%	
GlutaMAX	ThermoFisher /35050			10mL	1%	
NEAA	ThermoFisher /11140050			10mL	1%	
Insulin-Transferrin-Selenium (ITS-G)(100x)	ThermoFisher /41400045			10mL	1%	
Insulin	Sigma/I9278			500uL	5ug/mL	
MCSF (100ug/mL)	Peprotech/300-25			250uL	25ng/mL	
TGF-β1 (100ug/mL)	R&D Systems/240-B			0.5mL	50ng/mL	
IL-34 (100ug/mL)	Peprotech/200-34			1mL	100ng/mL	
CD200 (100ug/mL)	Acro / OX2-H5228			1mL	100ng/mL	
CX3CR (100ug/mL)	Peprotech / 300-31			1mL	100ng/mL	

FIG. 12 (CONT.)

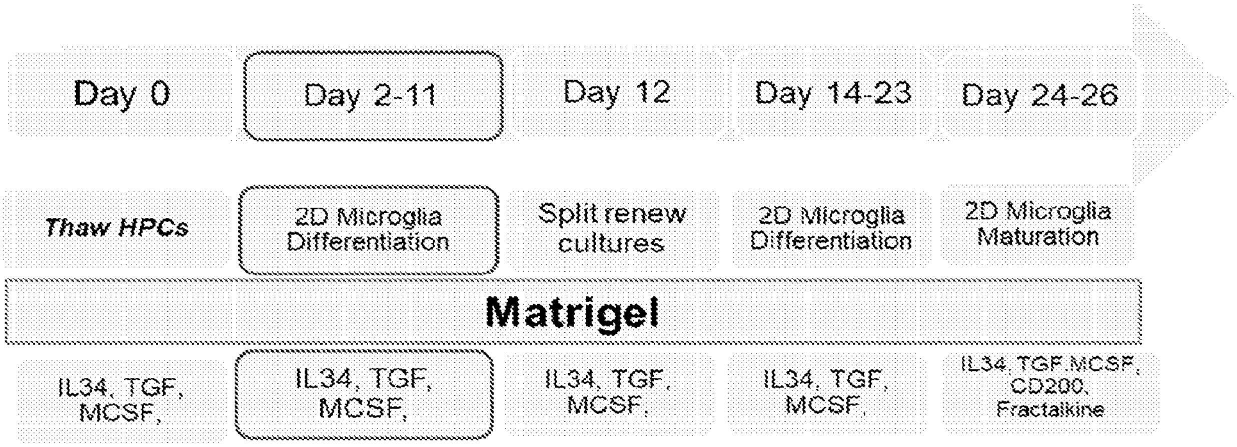
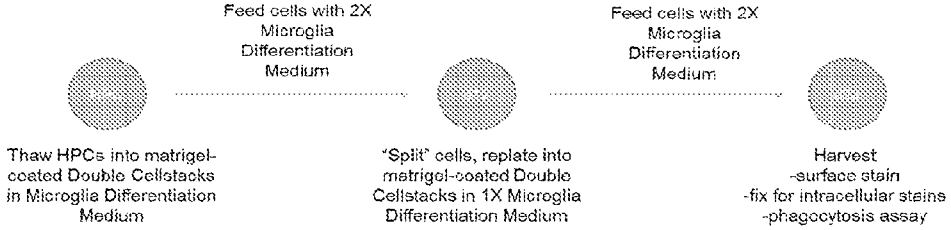


FIG. 13A


Feed cells with 2X Microglia Differentiation Medium
Feed cells with 2X Microglia Differentiation Medium
Thaw HPCs into matrigel-coated Double Cellstacks in Microglia Differentiation Medium
"Split" cells, replate into matrigel-coated Double Cellstacks in 1X Microglia Differentiation Medium
Harvest
-surface stain
-fix for intracellular stains
-phagocytosis assay

Microglia Differentiation Medium (MDM)

[illegible]	[illegible]	[illegible]
Microglia Basal Medium		
M-CSF	Peprotech / 300-25	25ng/mL
TGF-β1	R&D / 240-B	50ng/mL
IL-34	Peprotech / 200-34	100ng/mL

Microglia Differentiation Medium (2XMDM)

[illegible]	[illegible]	[illegible]
Microglia Basal Medium		
M-CSF	Peprotech / 300-25	50ng/mL
TGF-β1	R&D / 240-B	100ng/mL
IL-34	Peprotech / 200-34	200ng/mL

FIG. 13B

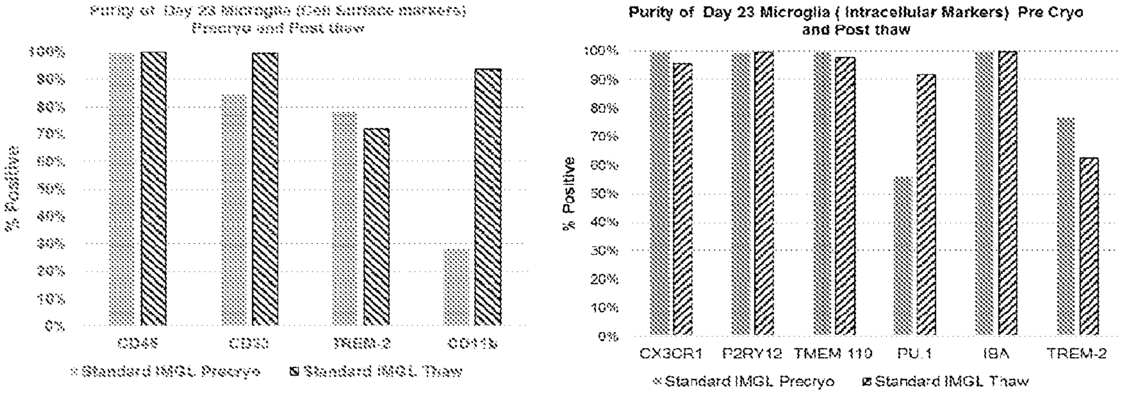
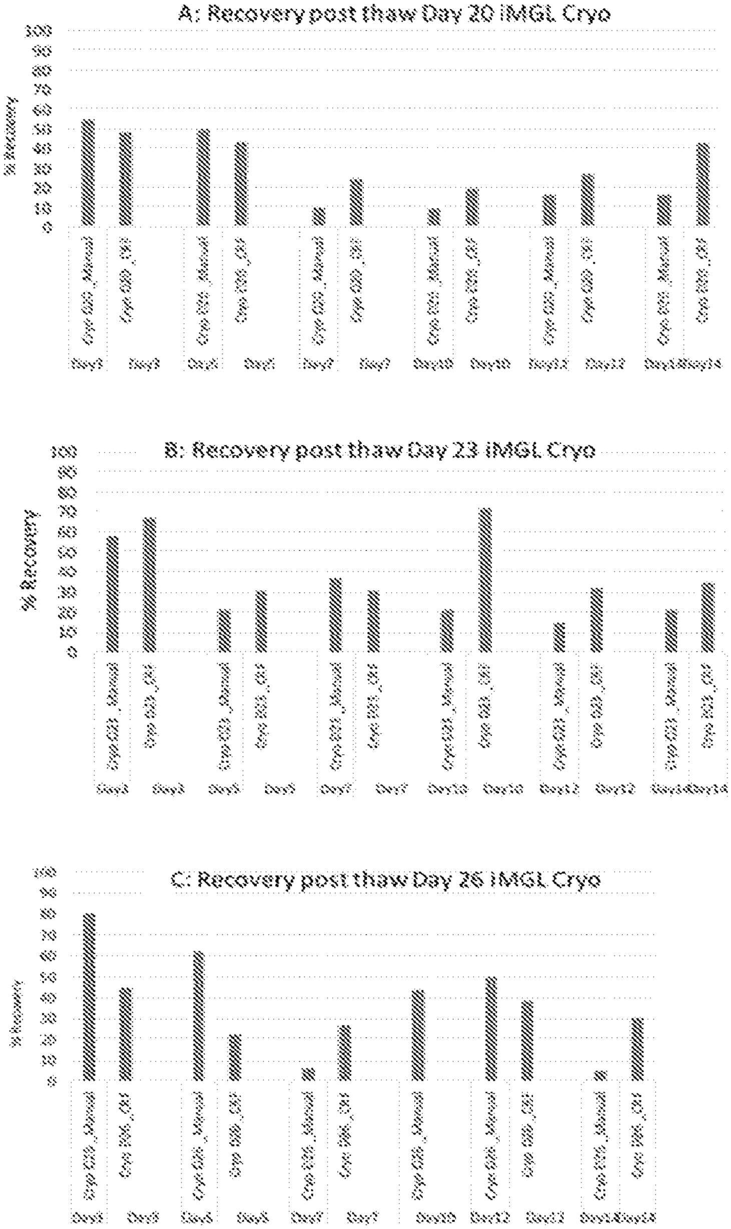

Purity of Day 23 Microglia (Cell Surface markers) Precryo and Post thaw
% Positive
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
CD45
CD33
TREM-2
CD11b
▨ Standard IMGL Precryo
▨ Standard IMGL Thaw
Purity of Day 23 Microglia (Intracellular Markers) Pre Cryo and Post thaw
% Positive
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
CX3CR1
P2RY12
TMEM 119
PU.1
IBA
TREM-2
▨ Standard IMGL Precryo
▨ Standard IMGL Thaw

FIG. 14


A: Recovery post thaw Day 20 iMGL Cryo
% Recovery
100
90
80
70
60
50
40
30
20
10
0
Day3
Day3
Day5
Day5
Day7
Day7
Day10
Day10
Day12
Day12
Day14
Day14
B: Recovery post thaw Day 23 iMGL Cryo
% Recovery
100
90
80
70
60
50
40
30
20
10
0
Day3
Day3
Day5
Day5
Day7
Day7
Day10
Day10
Day12
Day12
Day14
Day14
C: Recovery post thaw Day 26 iMGL Cryo
% Recovery
100
90
80
70
60
50
40
30
20
10
0
Day3
Day3
Day5
Day5
Day7
Day7
Day10
Day12
Day12
Day14
Day14

FIGS. 15A-15C

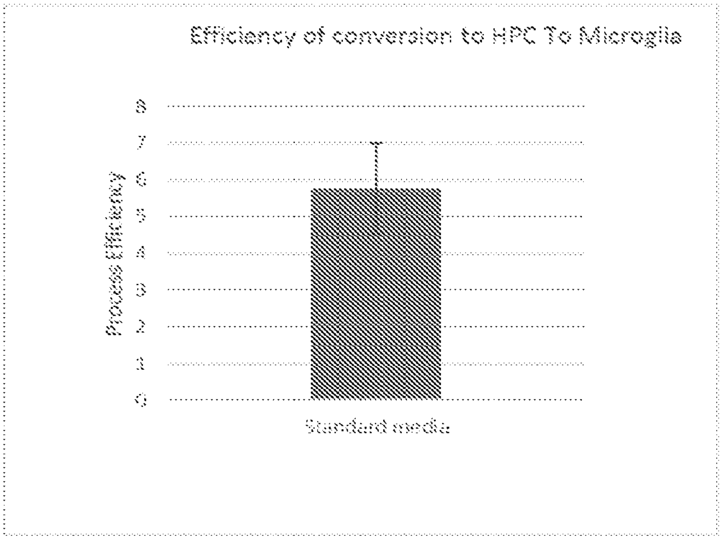
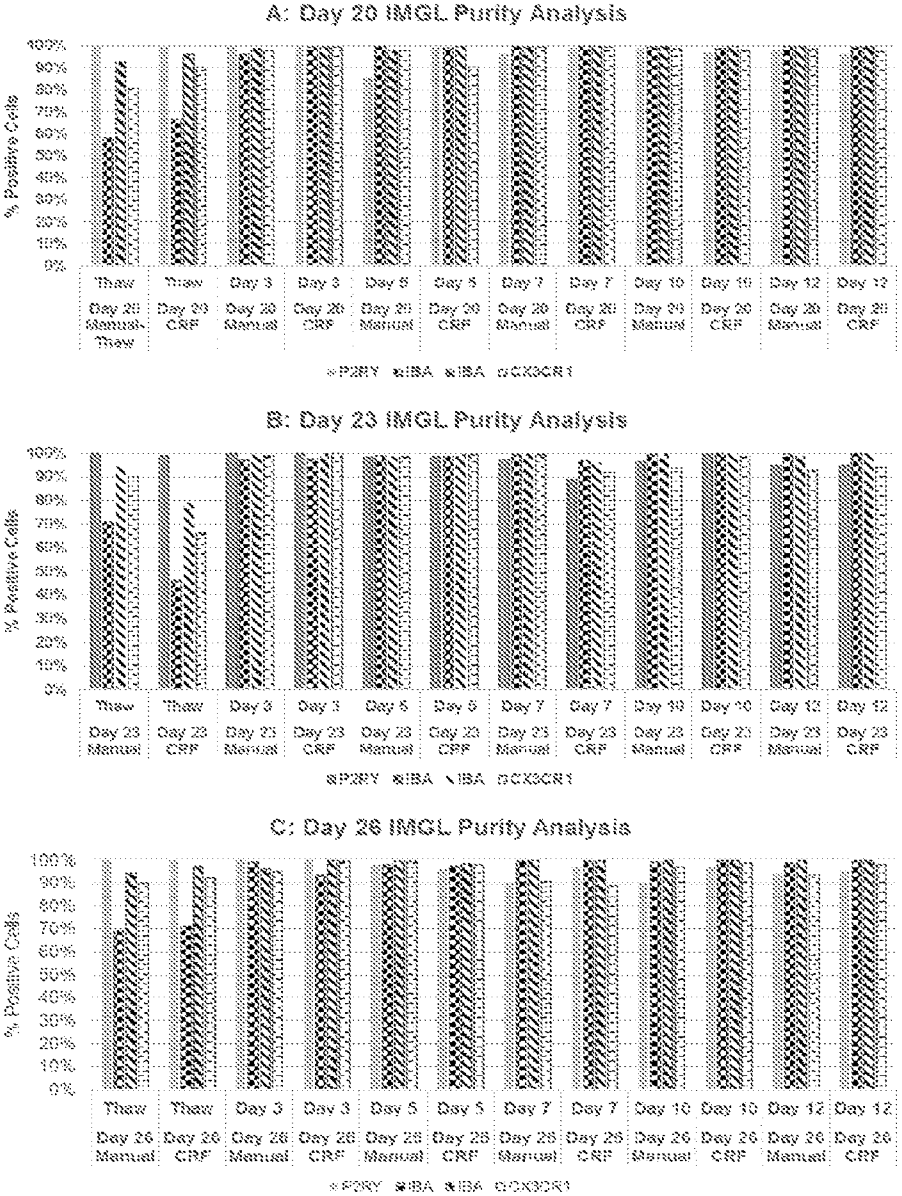
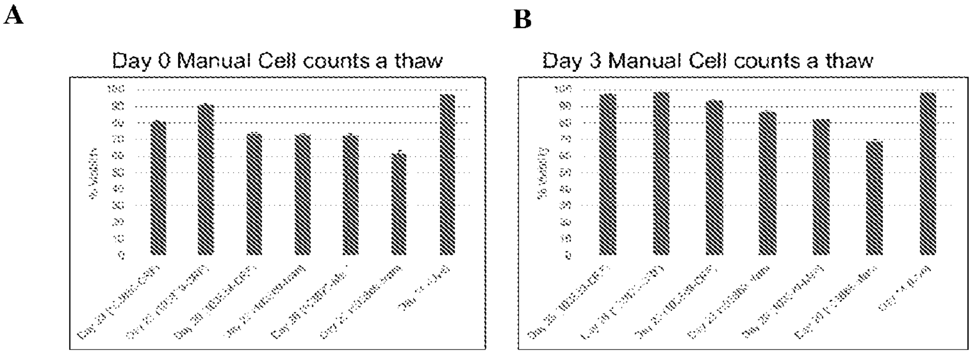

Efficiency of conversion to HPC To Microglia
Process Efficiency
8
7
6
5
4
3
2
1
0
Standard media

FIG. 16


A: Day 20 IMGL Purity Analysis
% Positive Cells
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Thaw
Thaw
Day 3
Day 3
Day 5
Day 5
Day 7
Day 7
Day 10
Day 10
Day 12
Day 12
Day 20 Manual-Thaw
Day 20 CRF
Day 20 Manual
Day 20 CRF
Day 20 Manual
Day 20 CRF
Day 20 Manual
Day 20 CRF
Day 20 Manual
Day 20 CRF
Day 20 Manual
Day 20 CRF
P2RY
IBA
IBA
CX3CR1
B: Day 23 IMGL Purity Analysis
% Positive Cells
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Thaw
Thaw
Day 3
Day 3
Day 5
Day 5
Day 7
Day 7
Day 10
Day 10
Day 12
Day 12
Day 23 Manual
Day 23 CRF
Day 23 Manual
Day 23 CRF
Day 23 Manual
Day 23 CRF
Day 23 Manual
Day 23 CRF
Day 23 Manual
Day 23 CRF
Day 23 Manual
Day 23 CRF
P2RY
IBA
IBA
CX3CR1
C: Day 26 IMGL Purity Analysis
% Positive Cells
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Thaw
Thaw
Day 3
Day 3
Day 5
Day 5
Day 7
Day 7
Day 10
Day 10
Day 12
Day 12
Day 26 Manual
Day 26 CRF
Day 26 Manual
Day 26 CRF
Day 26 Manual
Day 26 CRF
Day 26 Manual
Day 26 CRF
Day 26 Manual
Day 26 CRF
Day 26 Manual
Day 26 CRF
P2RY
IBA
IBA
CX3CR1

FIGS. 17A-17C



FIGS. 18A-18B

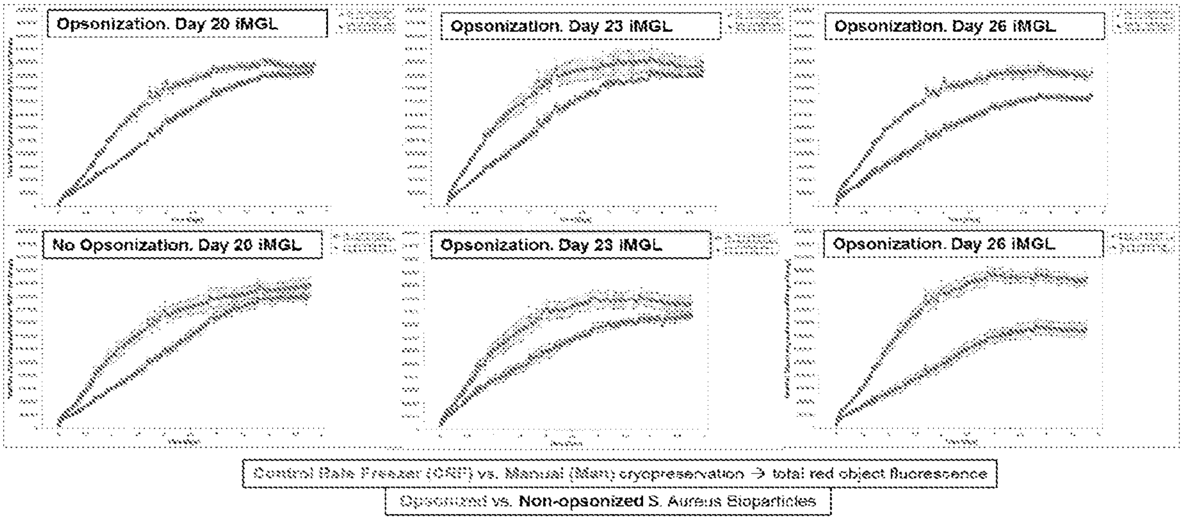


FIG. 19

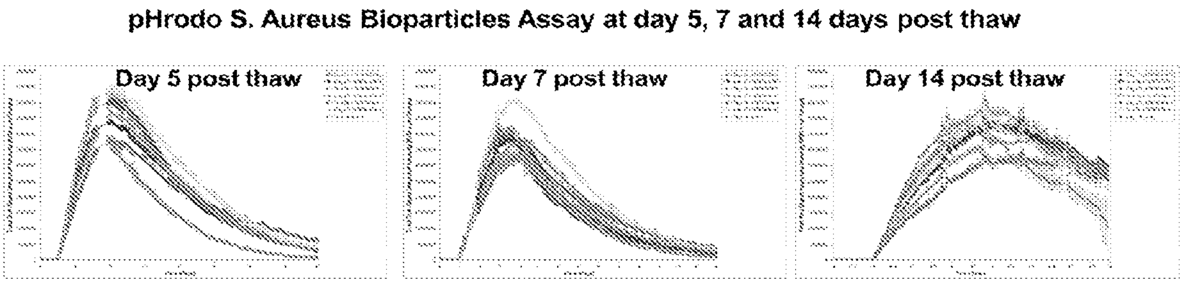

pHrodo S. Aureus Bioparticles Assay at day 5, 7 and 14 days post thaw
Day 5 post thaw
Day 7 post thaw
Day 14 post thaw

FIG. 20

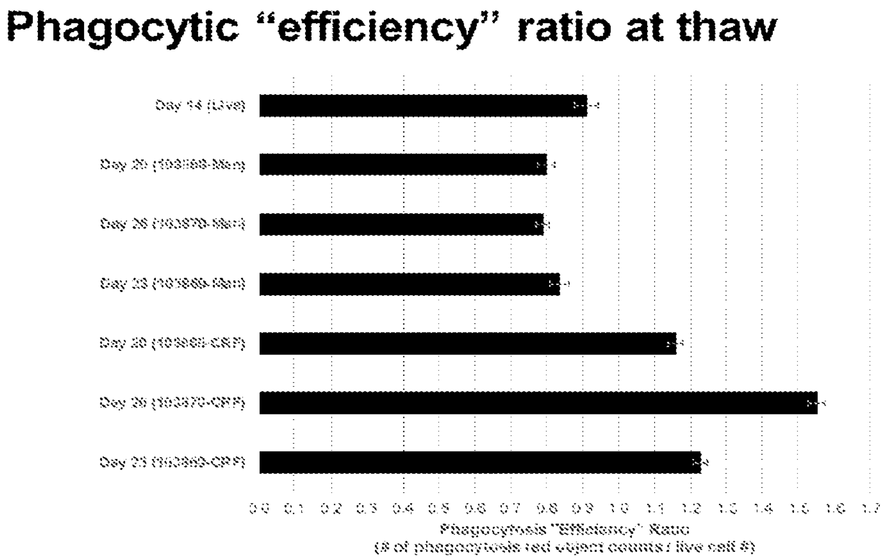

Phagocytic “efficiency” ratio at thaw
Day 14 (Live)
Day 20 (103868-Man)
Day 26 (103870-Man)
Day 23 (103869-Man)
Day 20 (103868-CRF)
Day 26 (103870-CRF)
Day 23 (103869-CRF)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
Phagocytosis "Efficiency" Ratio
(# of phagocytosis red object counts / live cell #)

FIG. 21

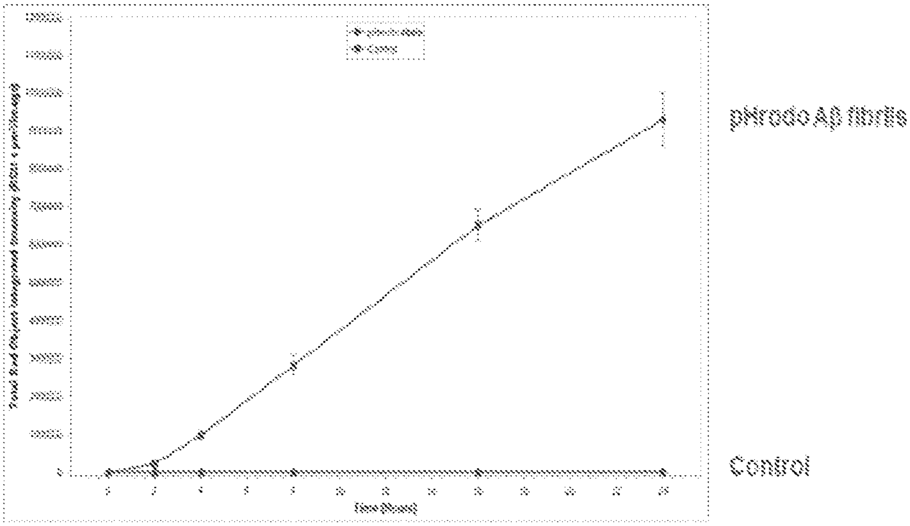


FIG. 22

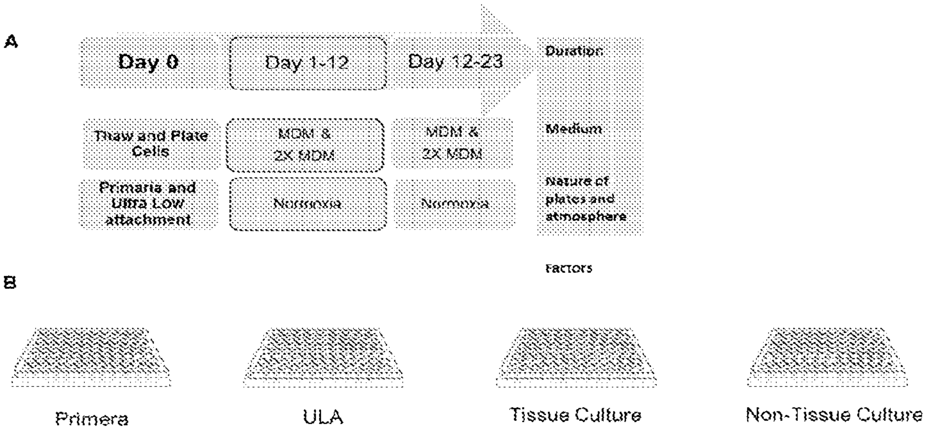
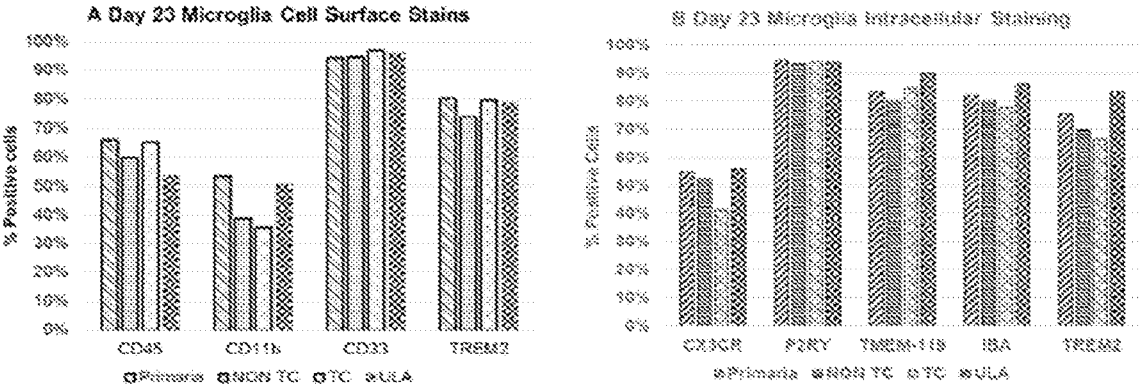


FIG. 23A-23B



FIGS. 24A-24B

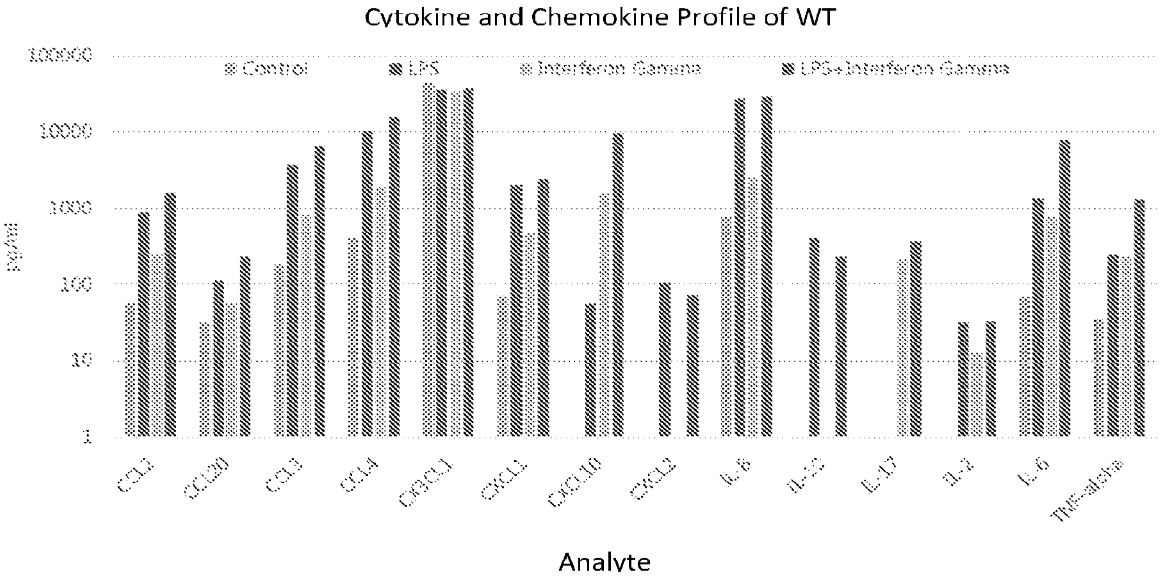


FIG. 25A

		Analyte (pg/mL)								
Cell Line	Condition	CCL2 / MCP-1	CCL20 / MIP-3 alpha	CCL4 / MIP-1 beta	CCL5 / RANTES	CX3CL1 / Fractalkine	CXCL1 / GRO alpha	CXCL10 / IP-10	CXCL2 / GRO beta	IL-8 / CXCL8
AHN	Control	245	3	361	3	52	15	13	2	276
	LPS	5456	1390	33788	214	450	8864	151	322	8515
SNCA	Control	279	15	570	23	54	18	16	2	701
	LPS	5798	1459	33860	396	489	6394	1274	284	8769
TREM2 HZ	Control	4431	8	248	0	126	42	117	6	1284
	LPS	6326	1737	75396	365	475	14521	733	571	8505
TREM2 HO	Control	5318	3	182	3	124	24	379	5	986
	LPS	6517	1115	101115	418	488	11981	1397	464	8703
MECP2	Control	4577	7	260	0	214	100	110	14	3670
	LPS	6362	1622	52414	537	503	15659	715	563	8430

FIG. 25B

	Average Percentage Purity Microglia Markers post thaw – Engineered Lines									
Line	CD33	CD45	TREM2	P2RY12	TMEM119	CX3CR1	IBA1	CD11c	CD11b	PU.1
SNCA	94	100	98	94	99	99	95	99	91	8
MECP2	97	100	81	91	98	98	96	99	87	50
TREM2 HZ	96	100	90	94	99	99	97	99	89	50
TREM2 HO	98	99	90	91	95	95	92	99	94	65

FIG. 26

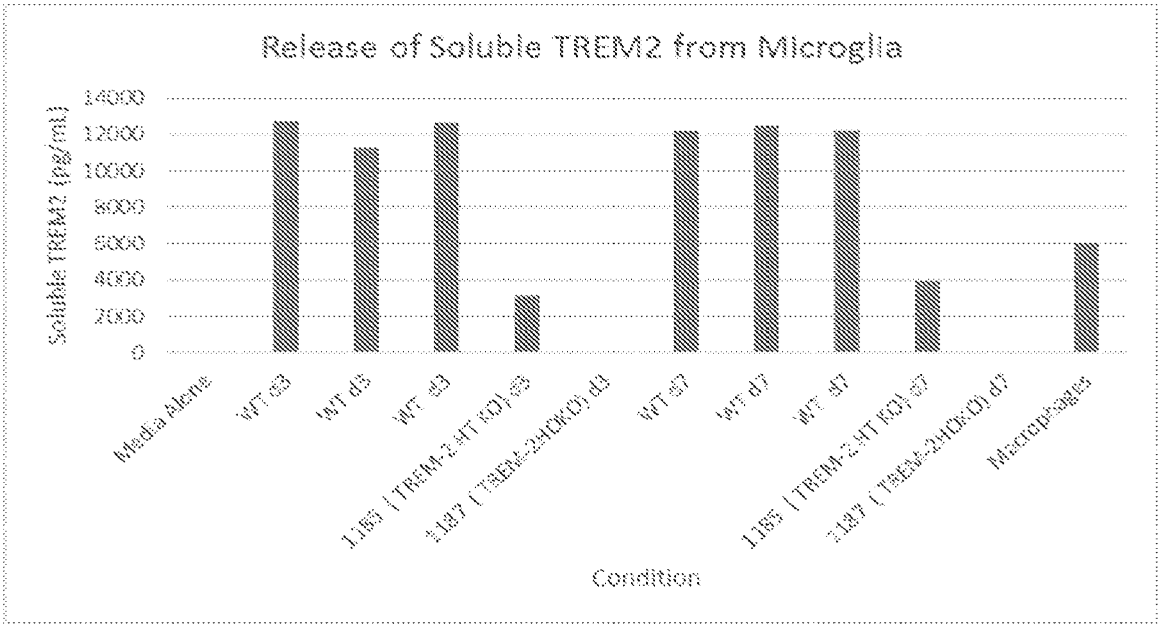

Release of Soluble TREM2 from Microglia
Soluble TREM2 (pg/mL)
14000
12000
10000
8000
6000
4000
2000
0
Media Alone
WT d3
WT d5
WT d3
1185 (TREM-2 HT KO) d3
1187 (TREM-2HO KO) d3
WT d7
WT d7
WT d7
1185 (TREM-2 HT KO) d7
1187 (TREM-2HO KO) d7
Macrophages
Condition

FIG. 27A

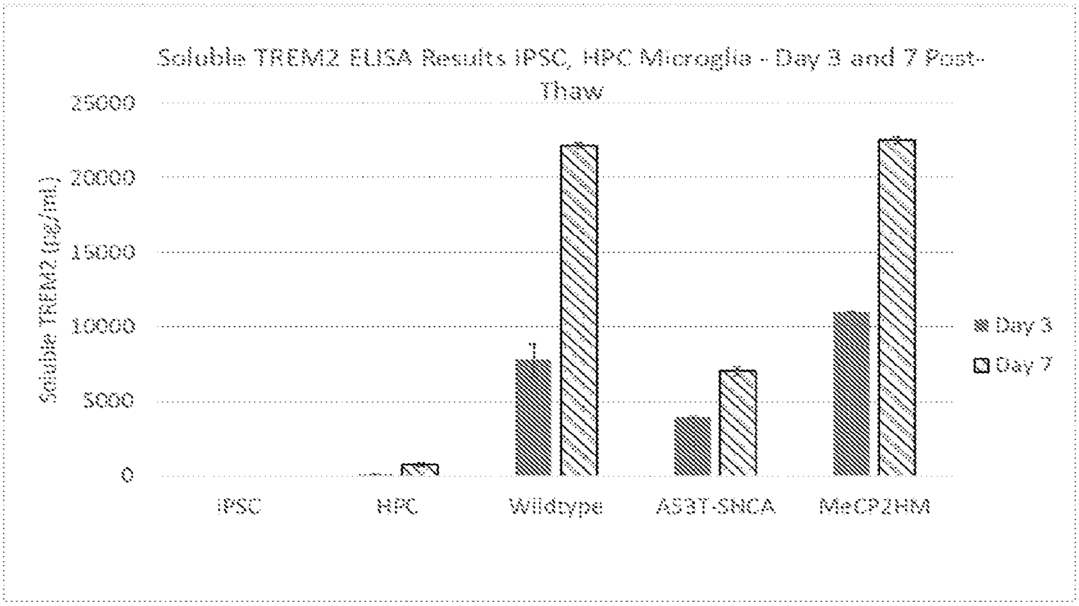
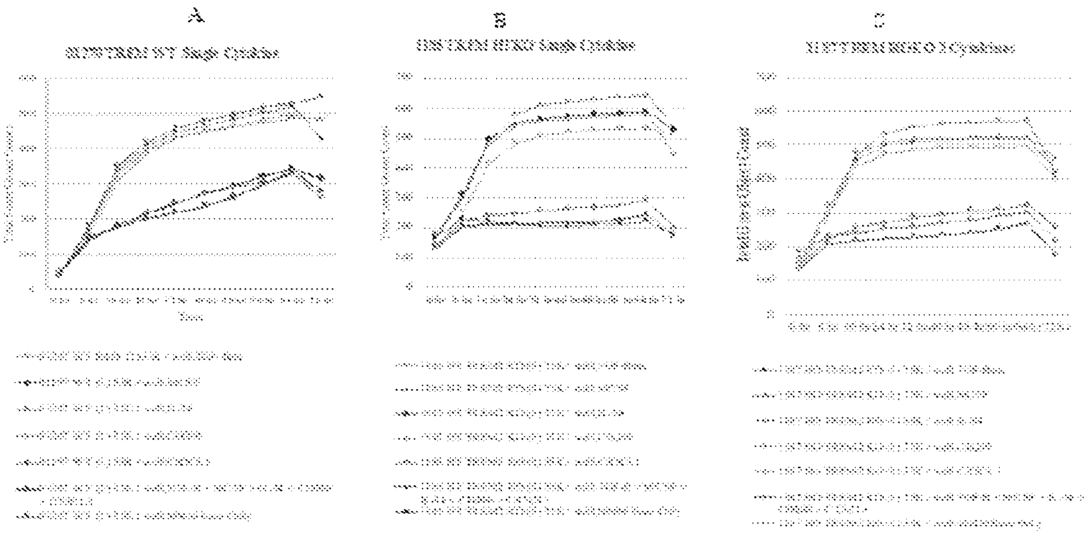


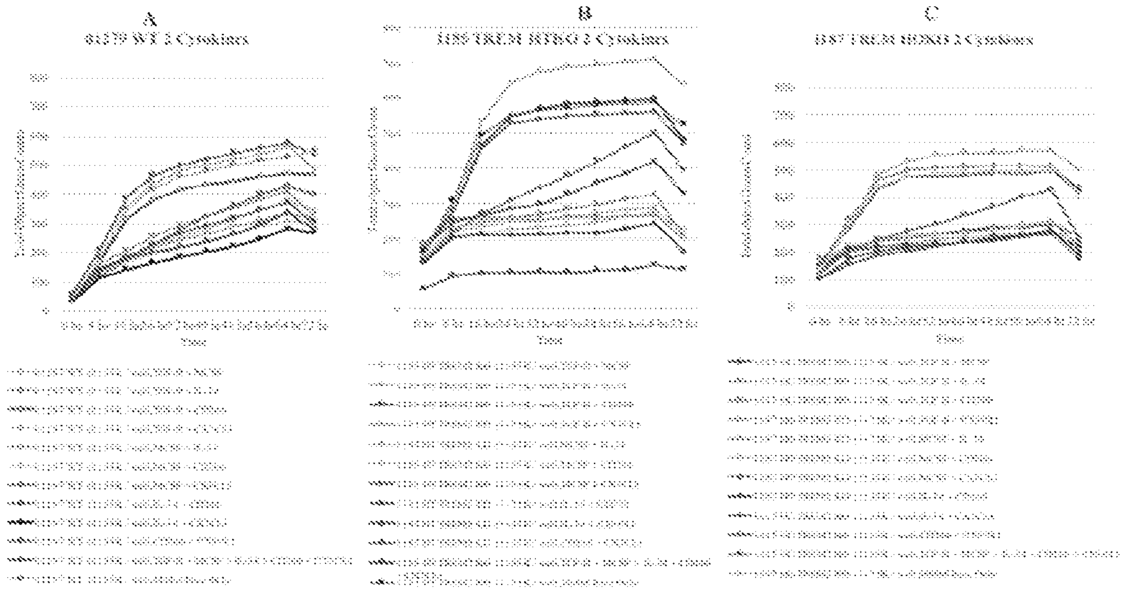
FIG. 27B

1	TGF-Beta
2	MCSF
3	IL-34
4	CD200
5	CX3CR4
6	TGF-B + MCSF
7	TGF-B + IL-34
8	TGF-B + CD200
9	TGF-B + CX3CR4
10	MCSF + IL-34
11	MCSF + CD200
12	MCSF + CX3CR4
13	IL-34 + CD200
14	IL-34 + CX3CR4
15	CD200 + CX3CR4
16	TGF-B + MCSF + IL-34
17	TGF-B + MCSF + CD200
18	TGF-B + MCSF + CX3CR4
19	TGF-B + IL-34 + CD200
20	TGF-B + IL-34 + CX3CR4
21	TGF-B + CD200 + CX3CR4
22	MCSF + IL-34 + CD200
23	MCSF + IL-34 + CX3CR4
24	MCSF + CD200 + CX3CR4
25	IL-34 + CD200 + CX3CR4
26	TGF-B + MCSF + IL-34 + CD200
27	TGF-B + MCSF + IL-34 + CX3CR4
28	TGF-B + MCSF + CD200 + CX3CR4
29	TGF-B + IL-34 + CD200 + CX3CR4
30	MCSF + IL-34 + CD200 + CX3CR4
31	TGF-B + MCSF + IL-34 + CD200 + CX3CR4
32	MMM Base Only

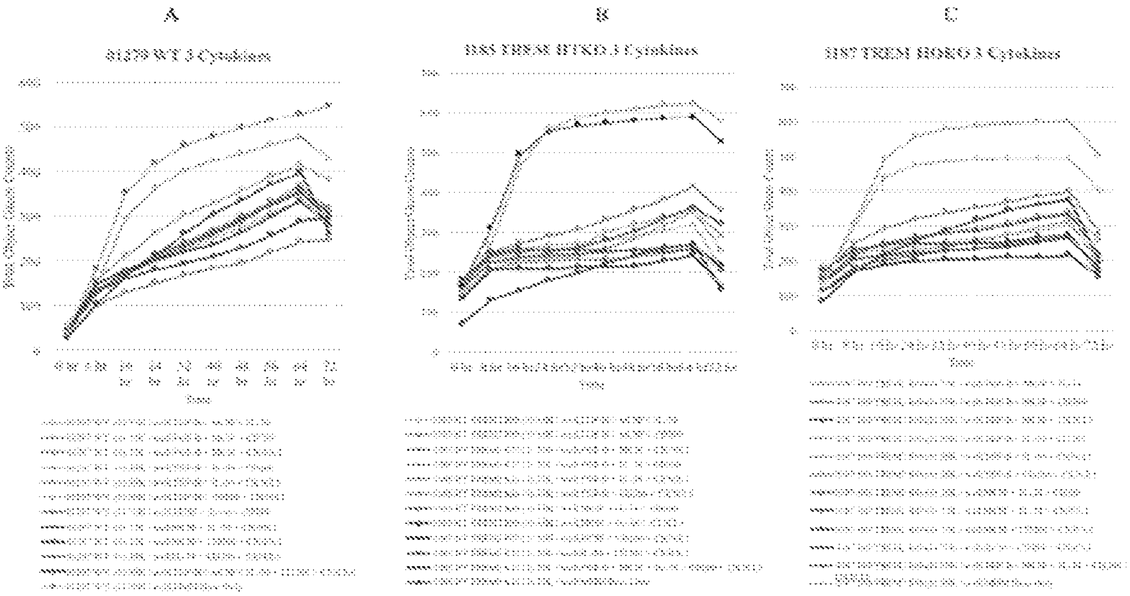
FIG. 28



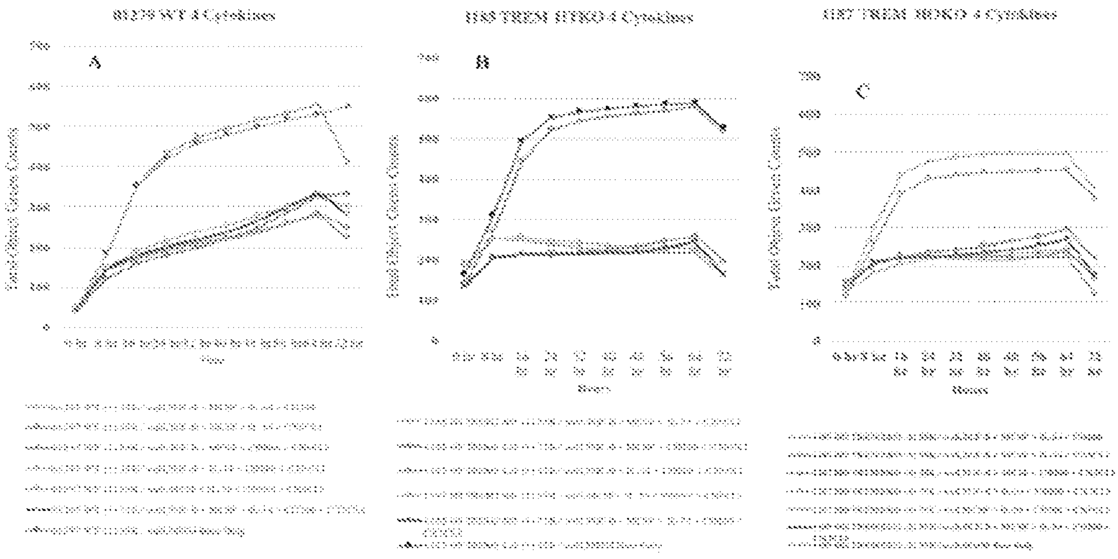
FIGS. 29A-29C



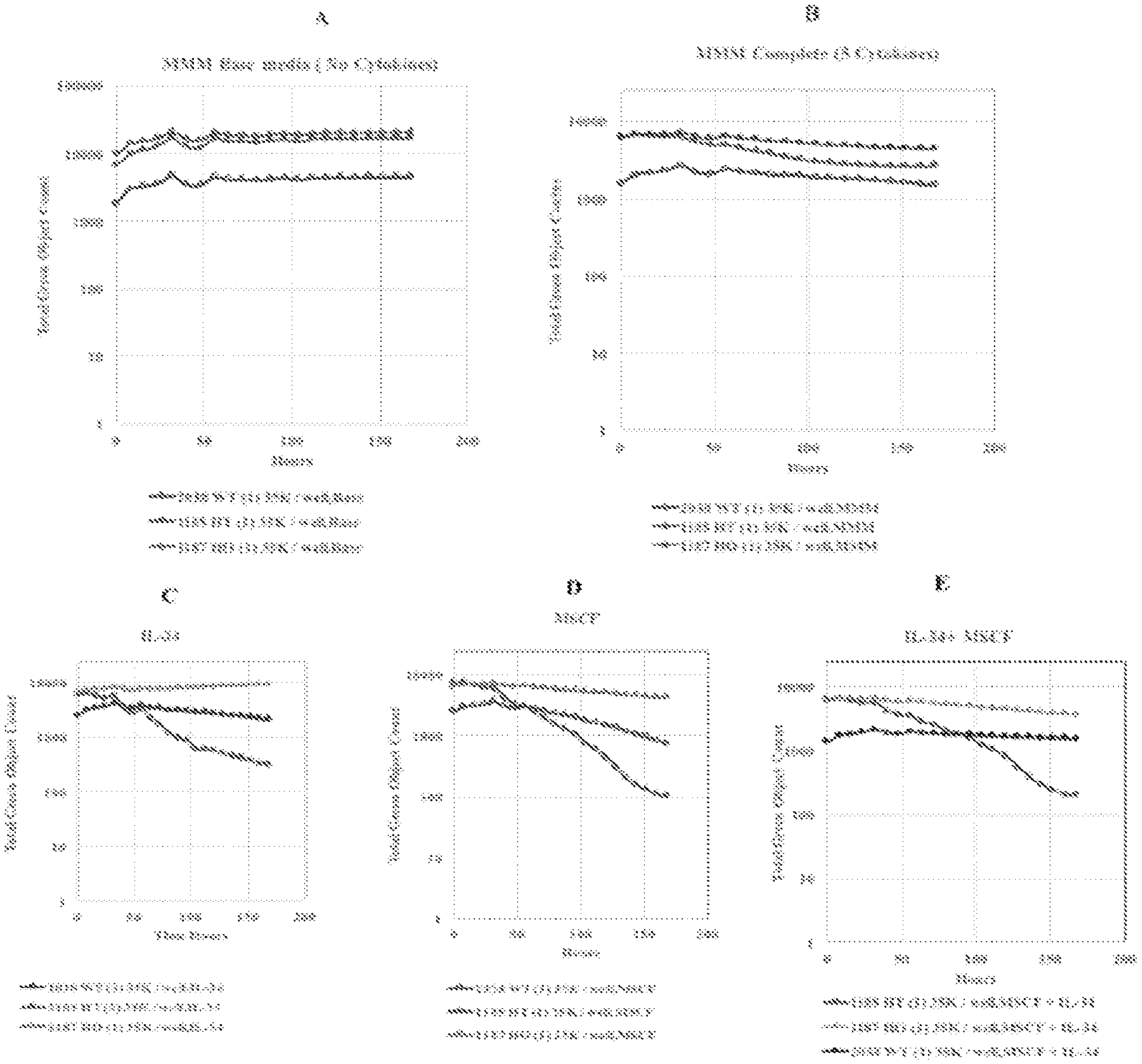
FIGS. 30A-30C


A
81279 WT 3 Cytokines
Time
B
1185 TREM IFTKD 3 Cytokines
Time
C
1187 TREM HOKO 3 Cytokines
Time

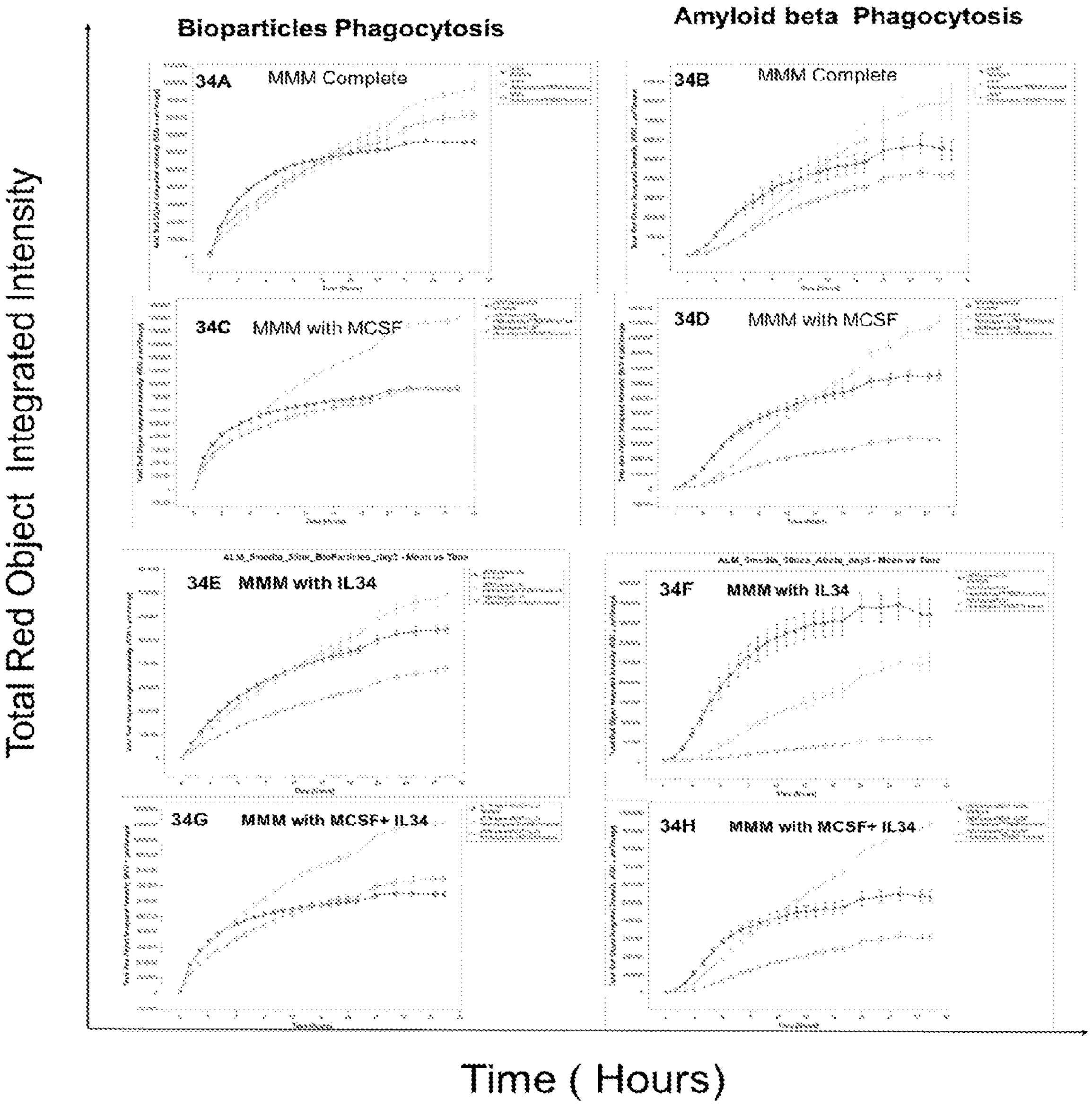
FIGS. 31A-31C


WT 4 Cytokines
A
TREM 4 Cytokines
B
TREM 4 Cytokines
C
700
600
500
400
300
200
100
0
Hours

FIGS. 32A-32C


A
MMM Base media (No Cytokines)
Total Green Object Count
100000
10000
1000
100
10
1
0
50
100
150
200
Hours
B
MMM Complete (5 Cytokines)
Total Green Object Count
10000
1000
100
10
1
0
50
100
150
200
Hours
C
IL-33
Total Green Object Count
10000
1000
100
10
1
0
50
100
150
200
Time Hours
D
MSCF
Total Green Object Count
10000
1000
100
10
1
0
50
100
150
200
Hours
E
IL-33+ MSCF
Total Green Object Count
10000
1000
100
10
1
0
50
100
150
200
Hours

FIGS. 33A-33E


Bioparticles Phagocytosis
Amyloid beta Phagocytosis
34A MMM Complete
34B MMM Complete
34C MMM with MCSF
34D MMM with MCSF
34E MMM with IL34
34F MMM with IL34
34G MMM with MCSF+ IL34
34H MMM with MCSF+ IL34
Total Red Object Integrated Intensity
Time (Hours)

FIGS. 34A-34H

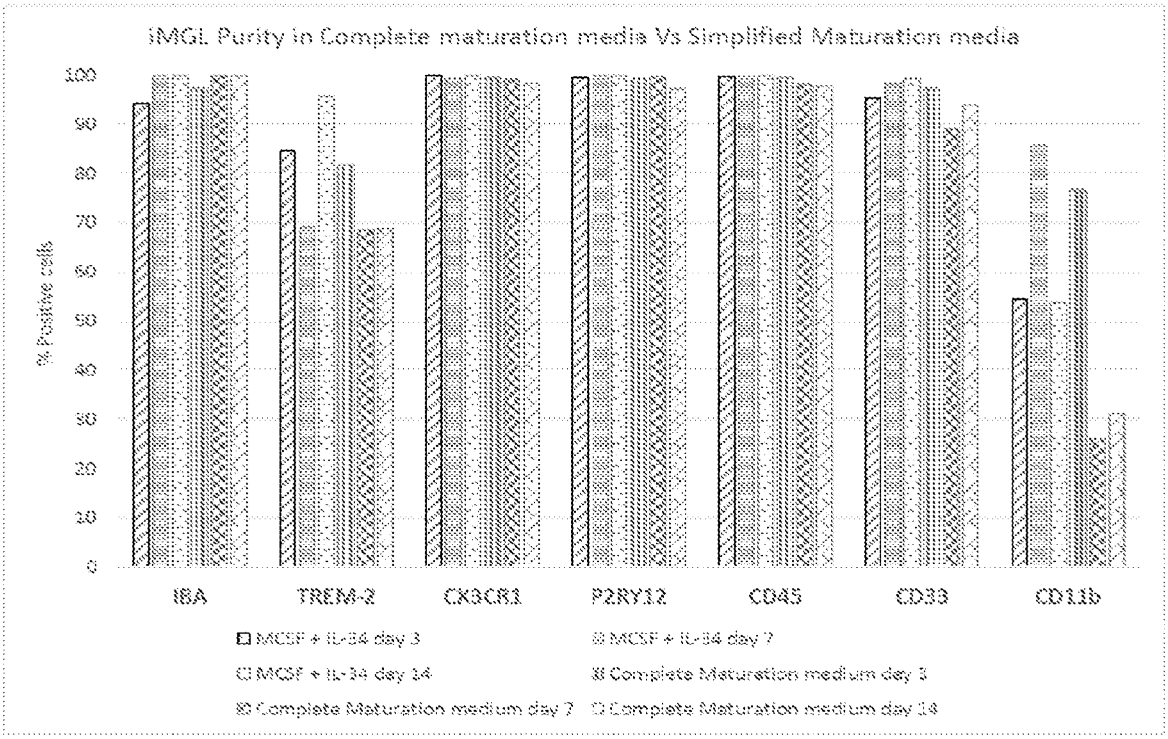

iMGL Purity in Complete maturation media Vs Simplified Maturation media
% Positive cells
100
90
80
70
60
50
40
30
20
10
0
IBA
TREM-2
CX3CR1
P2RY12
CD45
CD33
CD11b
MCSF + IL-34 day 3
MCSF + IL-34 day 7
MCSF + IL-34 day 14
Complete Maturation medium day 3
Complete Maturation medium day 7
Complete Maturation medium day 14

FIG. 35

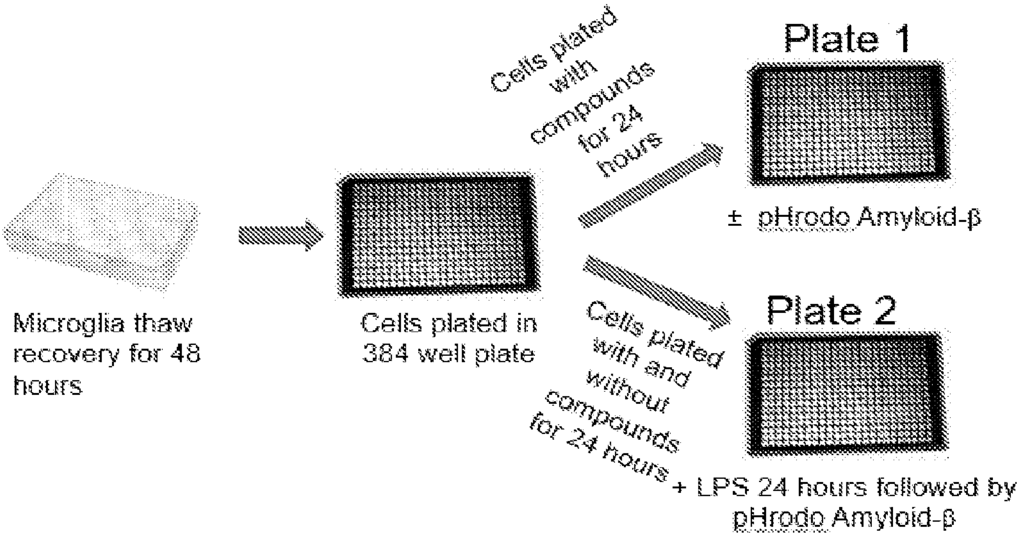
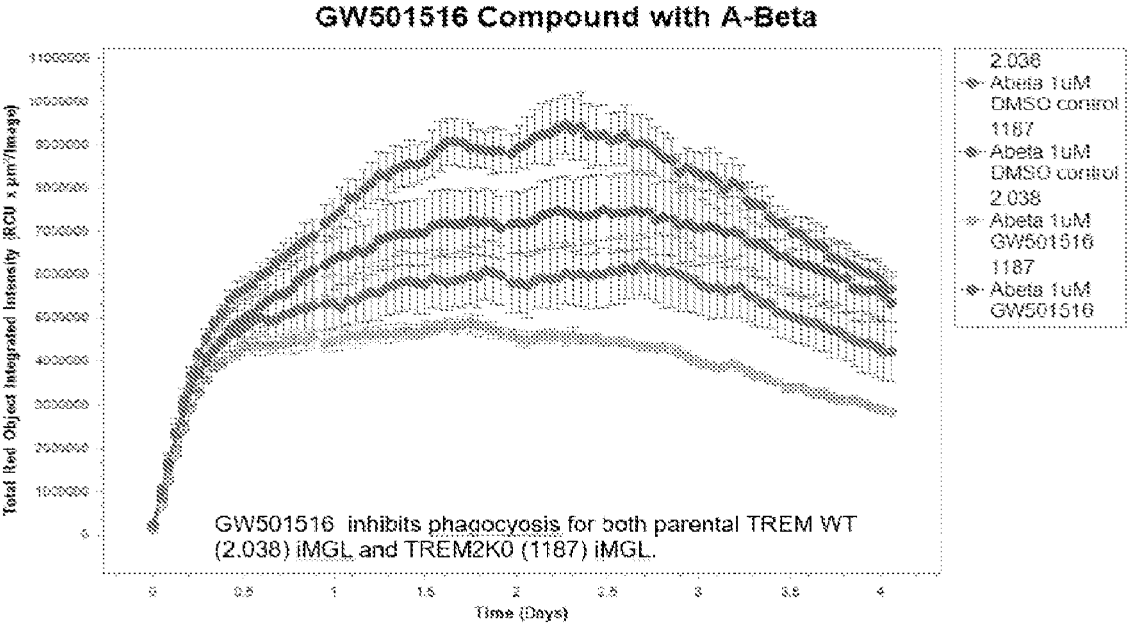

Microglia thaw recovery for 48 hours
Cells plated in 384 well plate
Cells plated with compounds for 24 hours
Plate 1
± pHrodo Amyloid-β
Cells plated with and without compounds for 24 hours
Plate 2
+ LPS 24 hours followed by pHrodo Amyloid-β

FIG. 36A

[illegible]		[illegible]
1	GW501516	PPAR-β agonist developed by GSK and Ligand Chemicals. Trophic effects on oligodendrocytes in vitro, and partial protection in experimental autoimmune encephalomyelitis (EAE). Anti inflammatory effects on BV2 microglia.
2	Leucettine L41	Pharmacological inhibitors of DYRKs (dual-specificity tyrosine phosphorylation regulated kinases) and CLKs (cdc2-like kinases)
3	Piceatannol	A natural stilbene and an analogue of Resveratrol. Antioxidative, anti- cancer properties, inhibitor of JAK-1 and Syk kinases.SRT1 activator and blocks adipogenesis. Included in the LOPAC collection.
4	Azeliragon	A potent inhibitor of DYRKs (dual-specificity tyrosine phosphorylation regulated kinases) and CLKs (cdc2-like kinases). Interacts also with GSK-3α/β, CK2 and PIM1. Neuroprotective against APP-induced cell death
5	J147	J 147 is a potent neuroprotective and neurotrophic compound that protects against neurotoxicity in cortical neurons in vitro (EC_{50} = 25 - 200 nM). Reduces soluble Aβ40 and Aβ42 levels and increases BDNF levels in the hippocampus in vivo. J147 prevents the loss of synaptic proteins and cognitive decline in a transgenic AD mouse model
6	Dibutyryl-cAMP	Dibutyryl-cAMP is a cell-permeable cyclic AMP (cAMP) analog that activates cAMP-dependent protein kinases. Stimulates neurite outgrowth. Can stimulate the activity of Na+, K+-ATPase (Na+,K+-pump). bring about morphological differentiation of astrocytes.
7	Isradipine	Cell-permeable, potent and selective voltage-gated L-type calcium channel blocker developed by Novartis. Displays anti atherosclerotic effects and improves endothelium-mediated nitric oxide-dependent vasodilation. Displays neuroprotective activity in rodent models.
8	Bexarotene	A highly potent and selective retinoid X receptor (RXR) agonist. Clinically useful antineoplastic agent for cutaneous T-cell lymphoma. Causes complete regression of mammary carcinoma. Bexarotene significantly increases clearance of soluble B-amyloid in a mouse Alzheimer's model and increases cognitive ability. Reverses apoE4-driven brain pathology and behavioral deficits in vivo.
9	SB-431542	A potent and selective inhibitor of the TGF-β 1 receptor ALK5 developed by GSK. Suppresses renewal in embryonic and induced pluripotent stem (iPS) cells and promotes their differentiation through its effect on Smad signaling pathway
10	GW2580	GW2580 is a selective inhibitor of cFMS blocking its ability to autophosphorylate colony-stimulating factor (CSF-1 or M-CSF), which promotes the survival, proliferation, and differentiation of macrophages.
11	SP600125	An anthrapyrazolone inhibitor of JNK that competes with ATP to inhibit the phosphorylation of c-Jun. It prevents the activation of inflammatory genes such as COX-2, IL-2 IFN-γ and TNF-α. Included in the LOPAC collection.
12	PP2	A selective inhibitor of Src-family tyrosine kinases with > 10,000-fold selectivity over ZAP-70 and JAK2. Compound discovered by Pfizer.
13	SB239063	A cell-permeable potent, reversible & ATP-competitive inhibitor of the α- and β-isoforms (IC_{50} = 44 nM), but not the γ- or δ-isoform (IC_{50} > 100 μM), of MAP kinase p38. SB 239063 reduces inflammatory cytokine production and is neuroprotective following oral administration in vivo.

FIG. 36B



GW501516 Compound with A-Beta

FIG. 37A

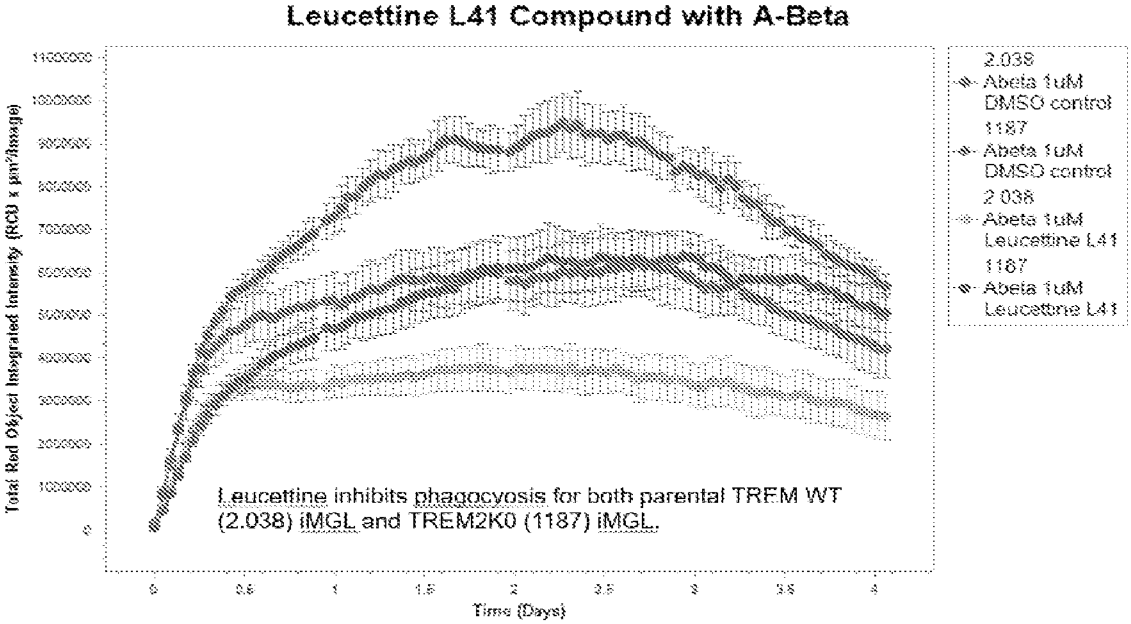
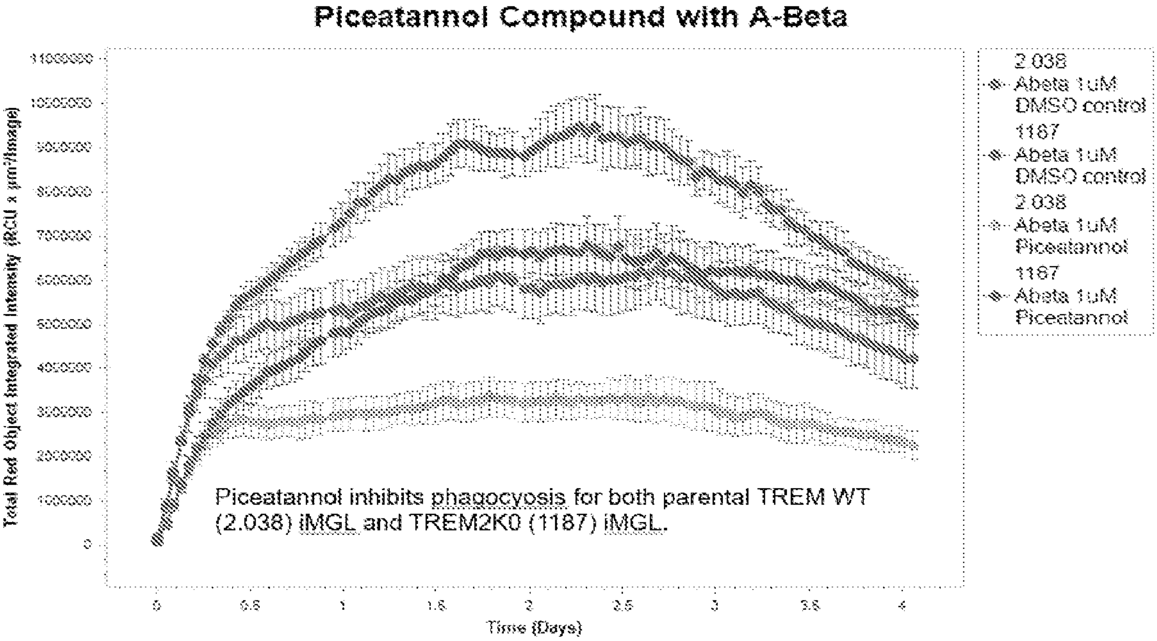
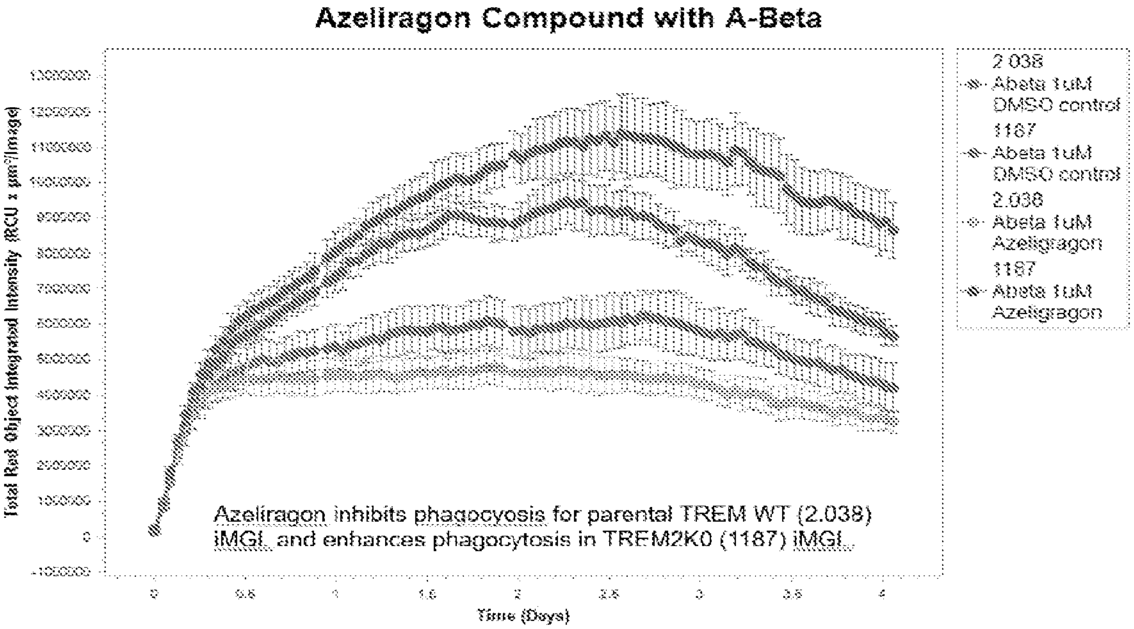


FIG. 37B


Piceatannol Compound with A-Beta

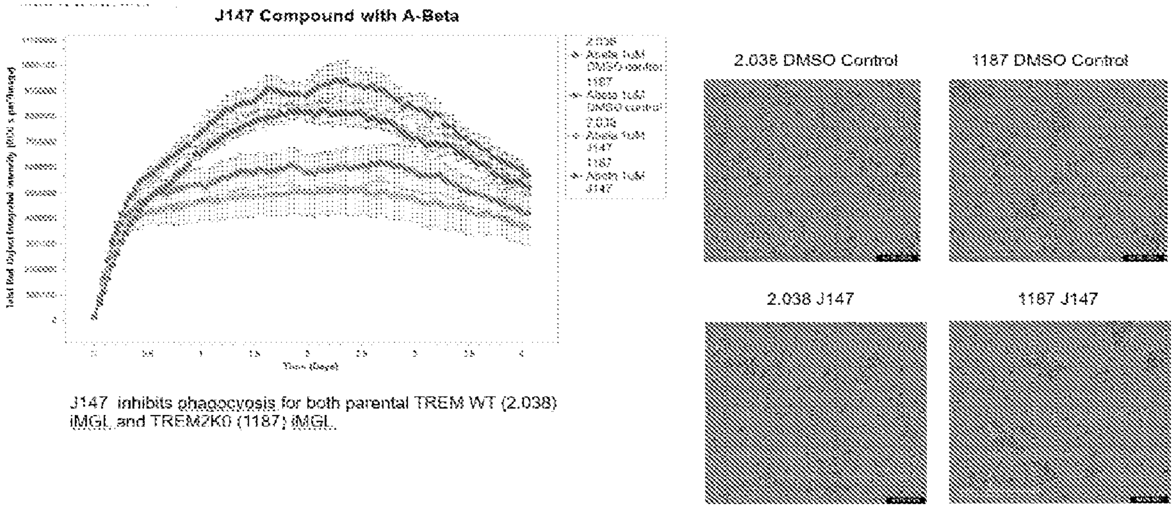
Piceatannol inhibits phagocyosis for both parental TREM WT (2.038) iMGL and TREM2K0 (1187) iMGL.

FIG. 37C


Azeliragon Compound with A-Beta

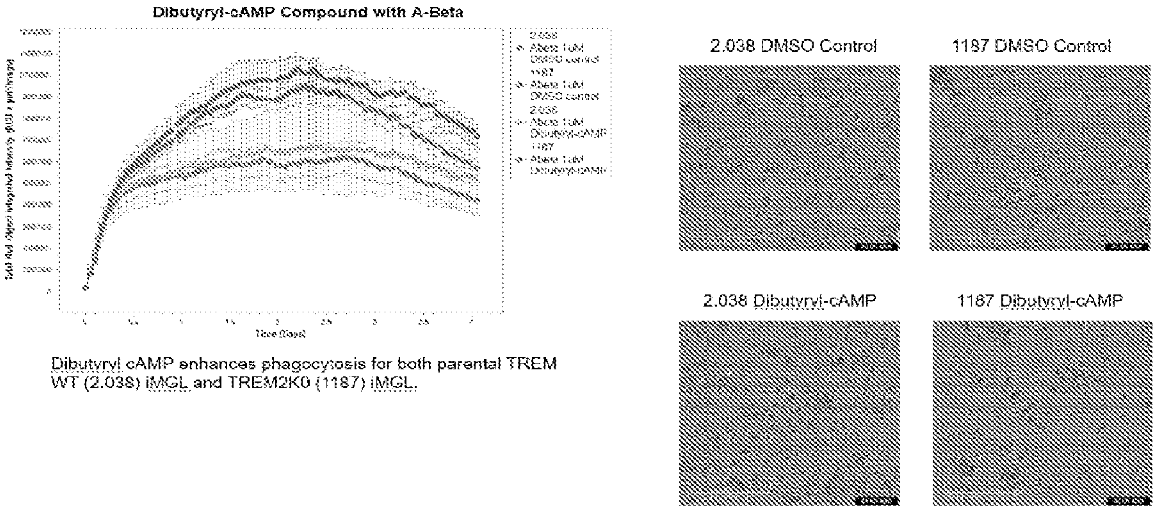
Azeliragon inhibits phagocysis for parental TREM WT (2.038) iMGL and enhances phagocytosis in TREM2K0 (1187) iMGL.

FIG. 37D



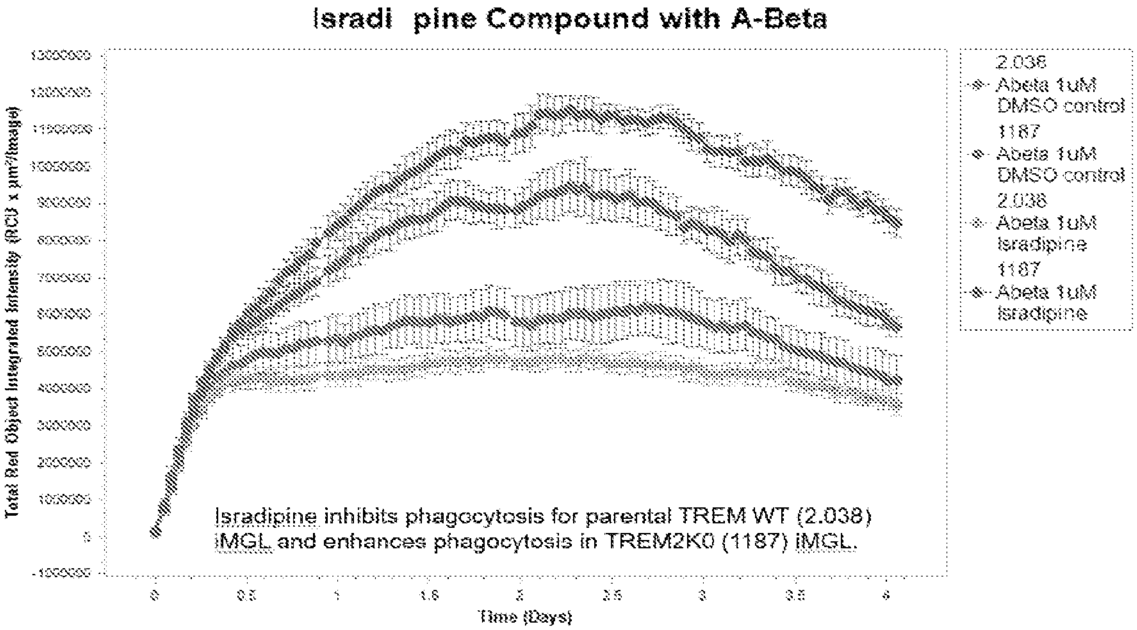
J147 inhibits phagocytosis for both parental TREM WT (2.038) iMGL and TREM2K0 (1187) iMGL.

FIG. 38A



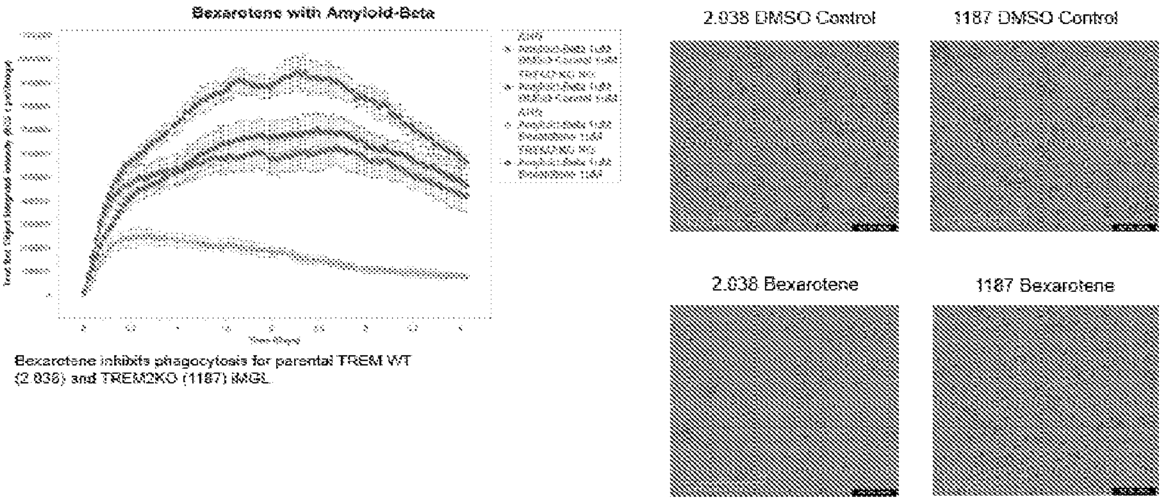
Dibutyryl cAMP enhances phagocytosis for both parental TREM WT (2.038) iMGL and TREM2K0 (1187) iMGL.

FIG. 38B



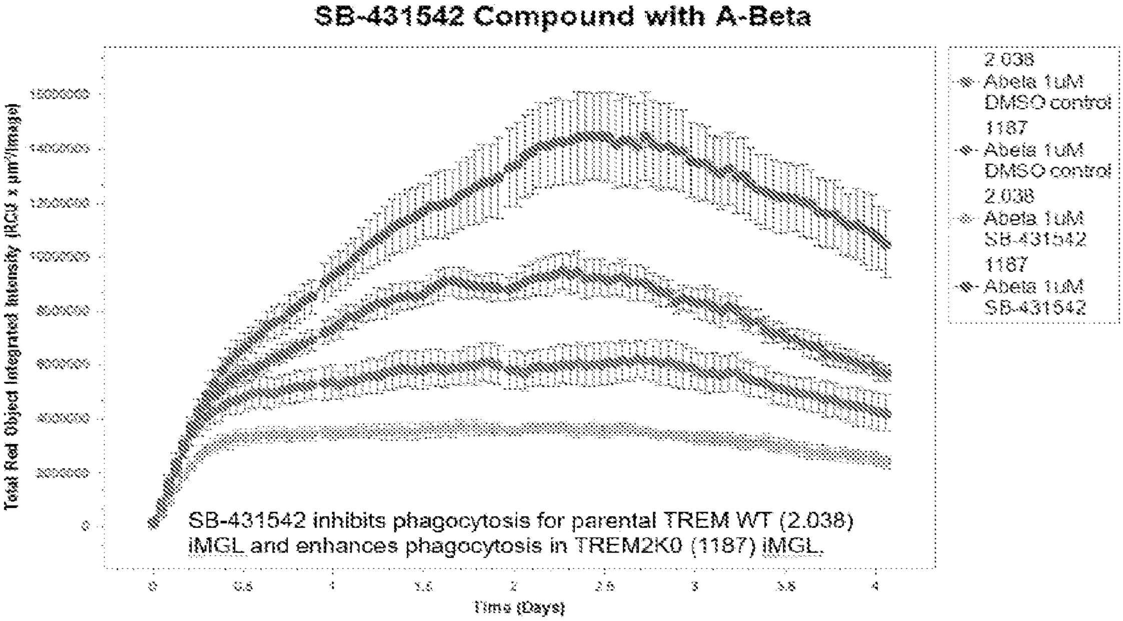
Isradipine inhibits phagocytosis for parental TREM WT (2.038) iMGL and enhances phagocytosis in TREM2K0 (1187) iMGL.

FIG. 38C



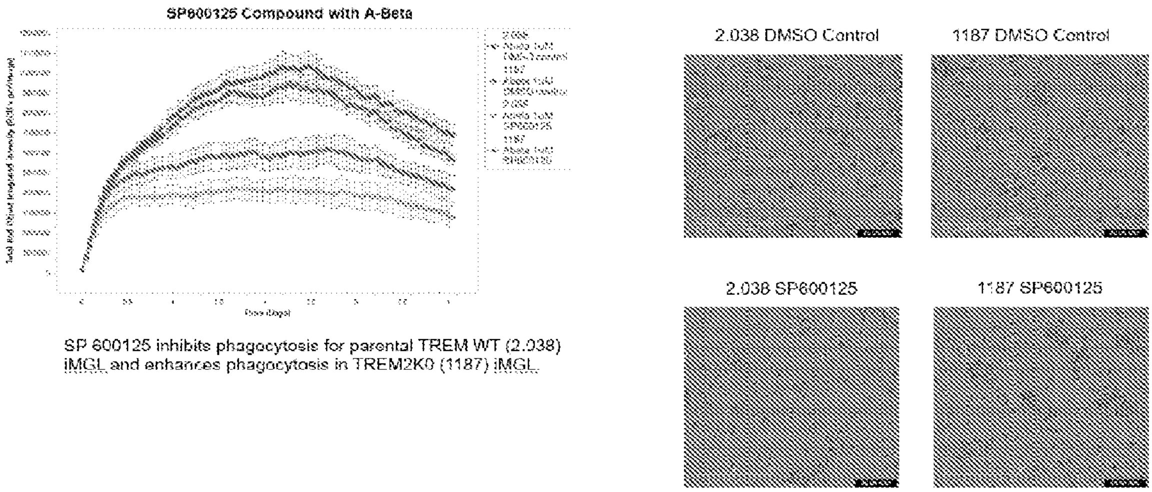
Bexarotene inhibits phagocytosis for parental TREM WT (2.038) and TREM2KO (1187) iMGL.

FIG. 38D



SB-431542 inhibits phagocytosis for parental TREM WT (2.038) iMGL and enhances phagocytosis in TREM2K0 (1187) iMGL.

FIG. 39A



SP 600125 inhibits phagocytosis for parental TREM WT (2.038) iMGL and enhances phagocytosis in TREM2K0 (1187) iMGL.

FIG. 39B

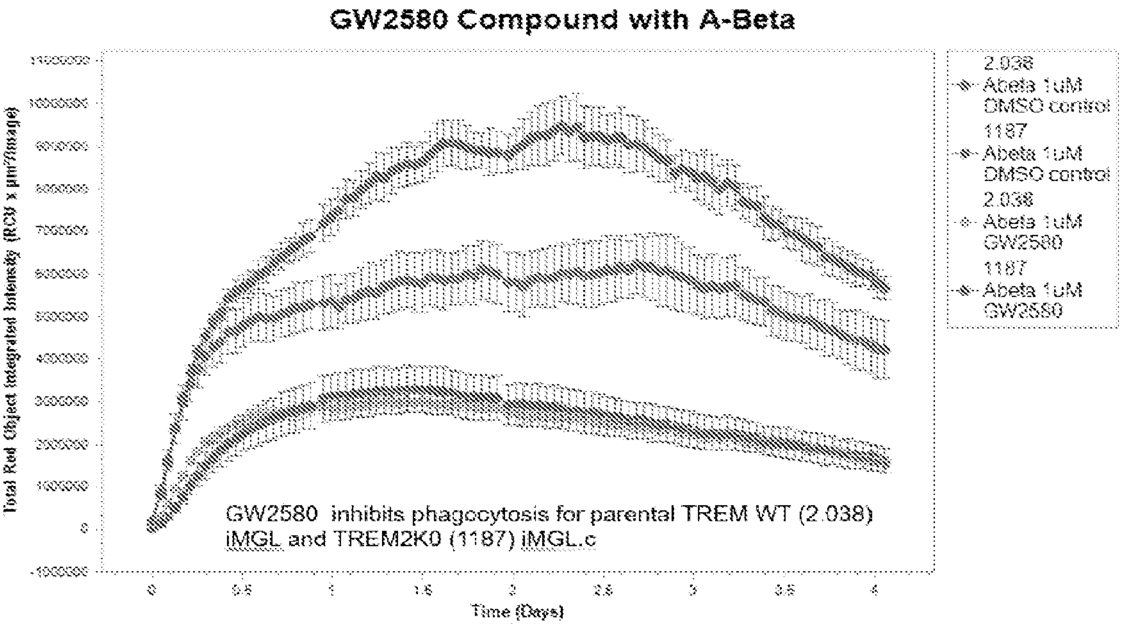


FIG. 39C

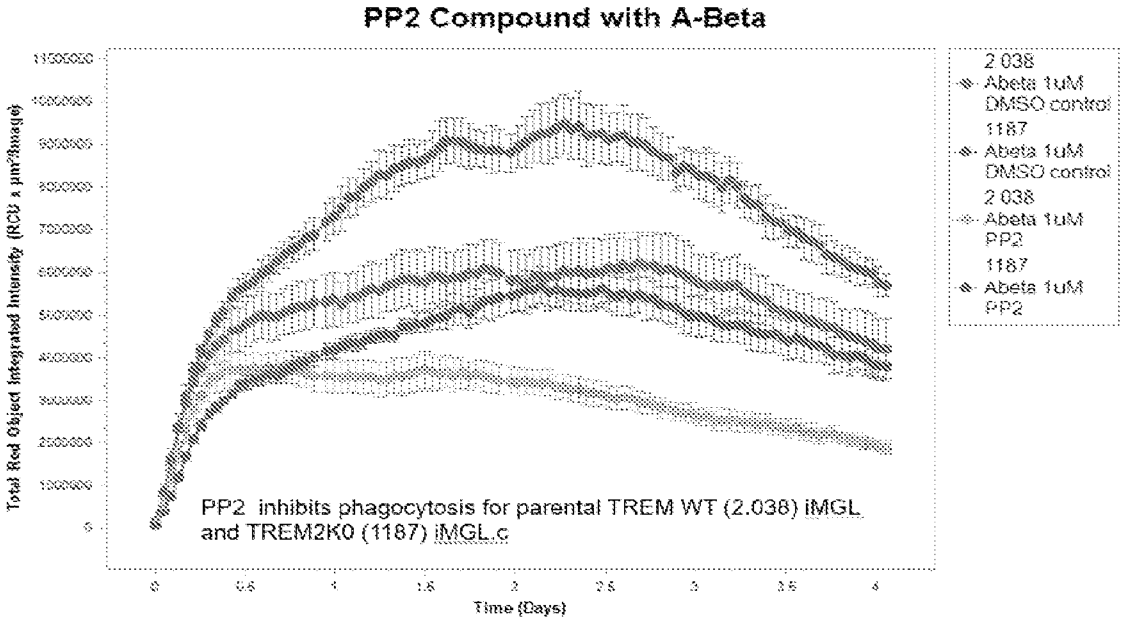
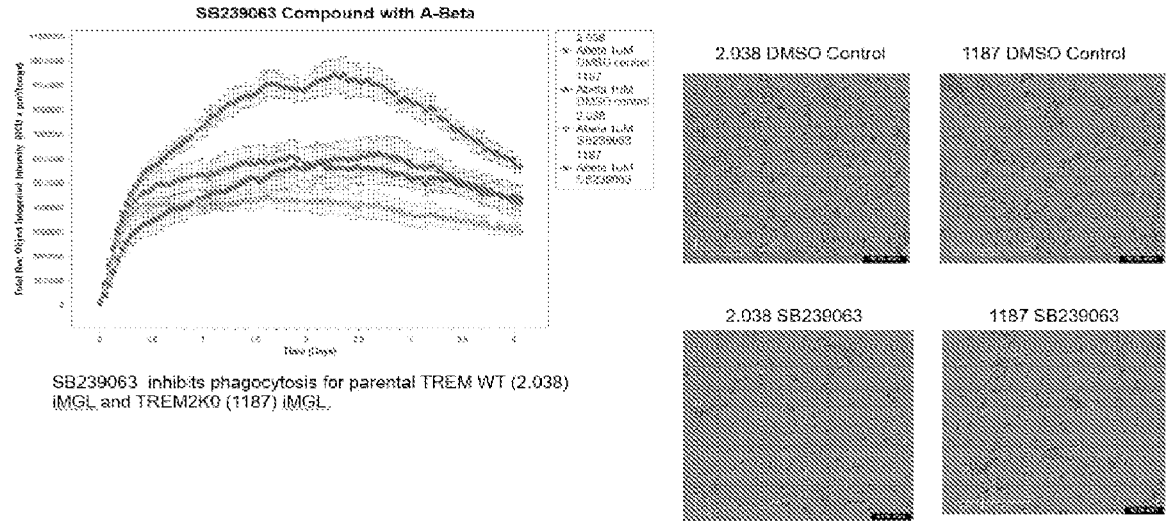
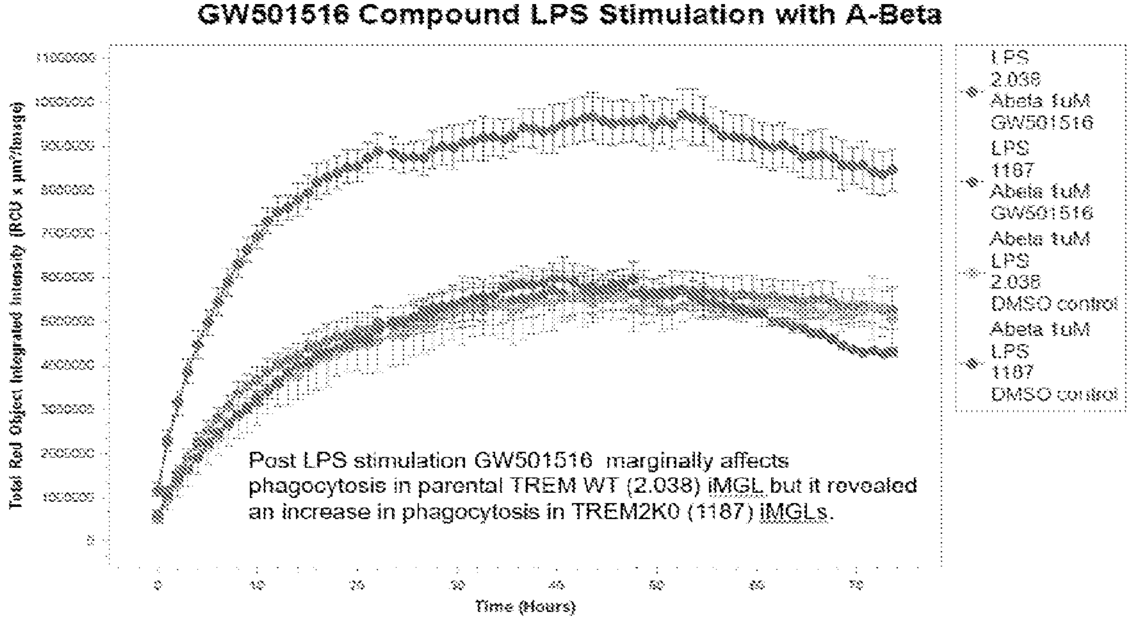


FIG. 39D



SB239063 inhibits phagocytosis for parental TREM WT (2.038) iMGL and TREM2K0 (1187) iMGL.

FIG. 39E



Post LPS stimulation GW501516 marginally affects phagocytosis in parental TREM WT (2.038) iMGL but it revealed an increase in phagocytosis in TREM2K0 (1187) iMGLs.

FIG. 40A

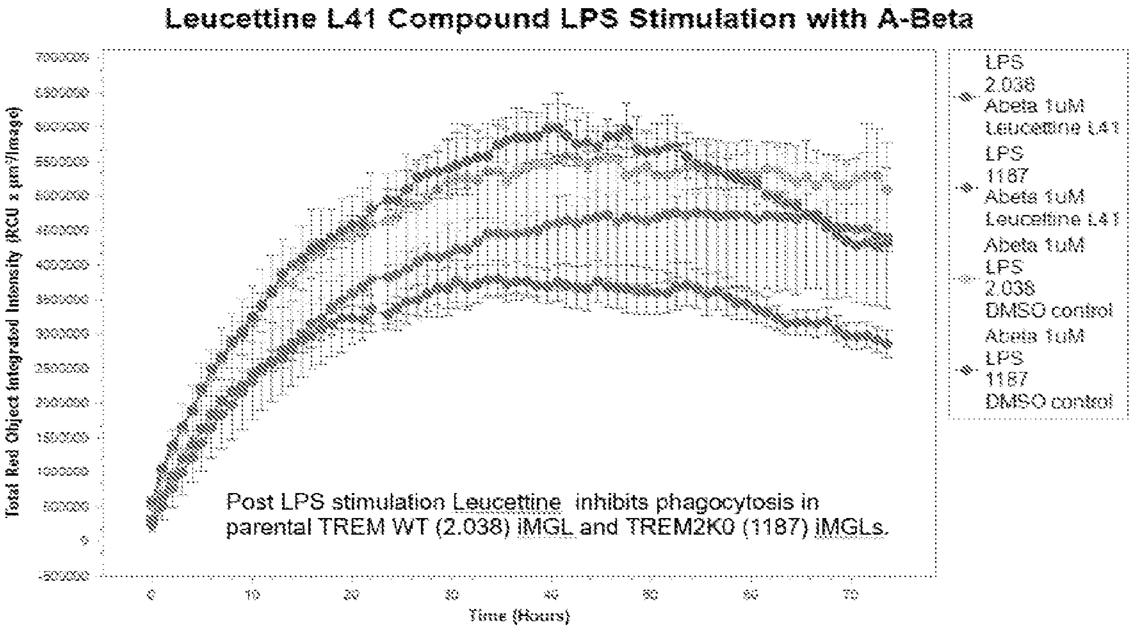

Leucettine L41 Compound LPS Stimulation with A-Beta
Total Red Object Integrated Intensity (RCU x µm²/Image)
7000000
6500000
6000000
5500000
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
-500000
0
10
20
30
40
50
60
70
Time (Hours)
LPS 2.038 Abeta 1uM Leucettine L41
LPS 1187 Abeta 1uM Leucettine L41
Abeta 1uM LPS 2.038 DMSO control
Abeta 1uM LPS 1187 DMSO control
Post LPS stimulation Leucettine inhibits phagocytosis in parental TREM WT (2.038) iMGL and TREM2K0 (1187) iMGLs.

FIG. 40B

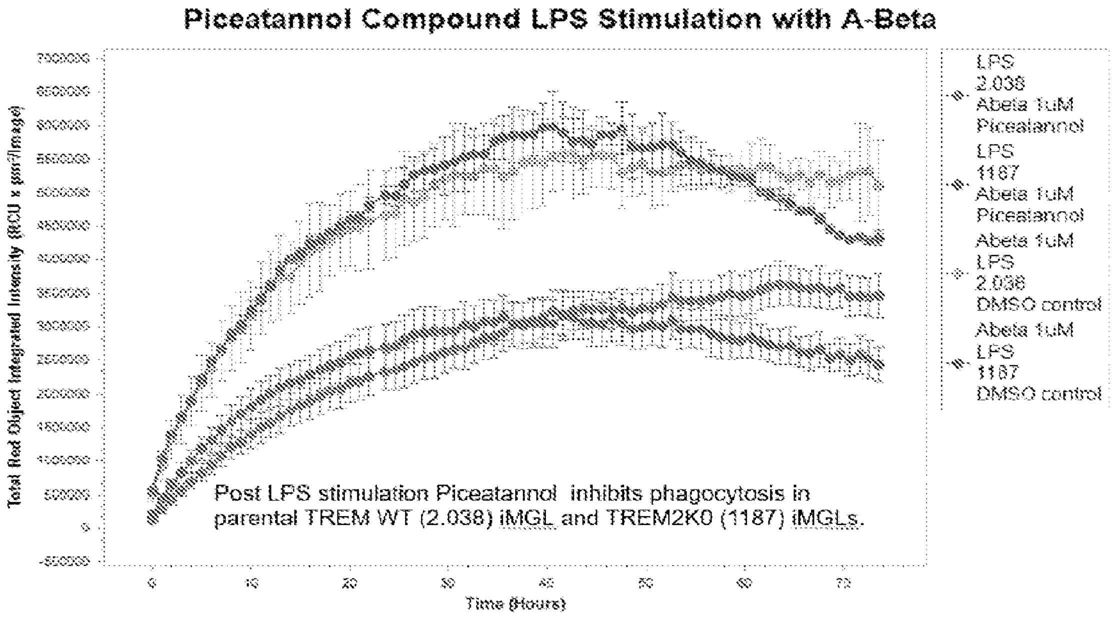
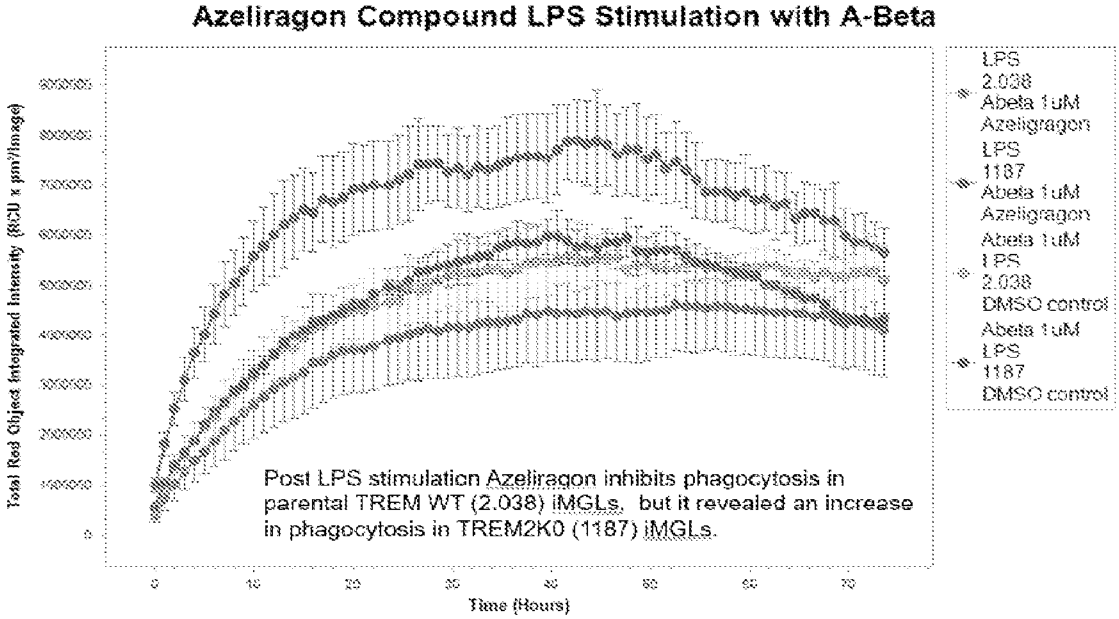
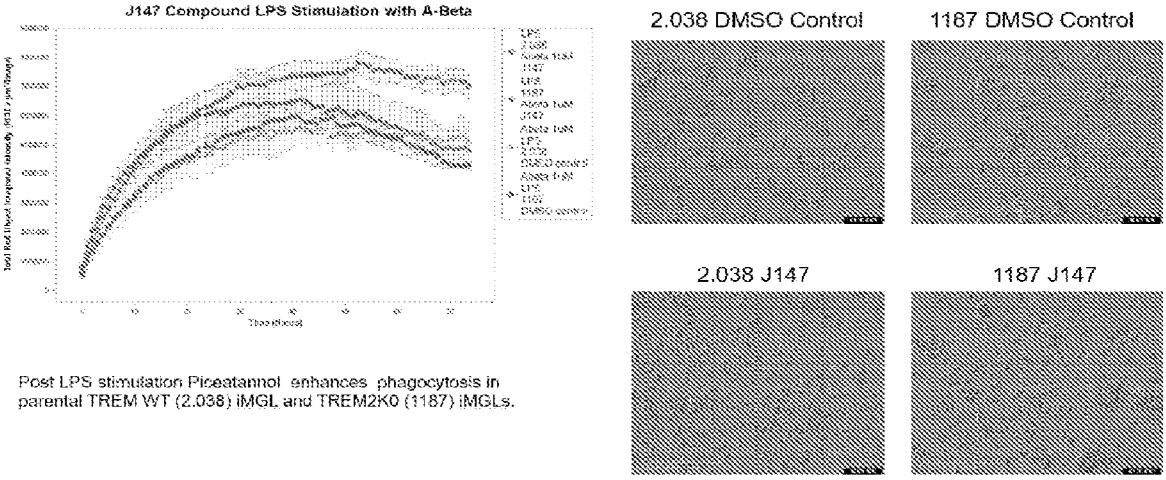

Piceatannol Compound LPS Stimulation with A-Beta
Total Red Object Integrated Intensity (RCU x µm²/Image)
7000000
6500000
6000000
5500000
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
-500000
0
10
20
30
40
50
60
70
Time (Hours)
LPS 2.038 Abeta 1uM Piceatannol
LPS 1187 Abeta 1uM Piceatannol
Abeta 1uM LPS 2.038 DMSO control
Abeta 1uM LPS 1187 DMSO control
Post LPS stimulation Piceatannol inhibits phagocytosis in parental TREM WT (2.038) iMGL and TREM2K0 (1187) iMGLs.

FIG. 40C



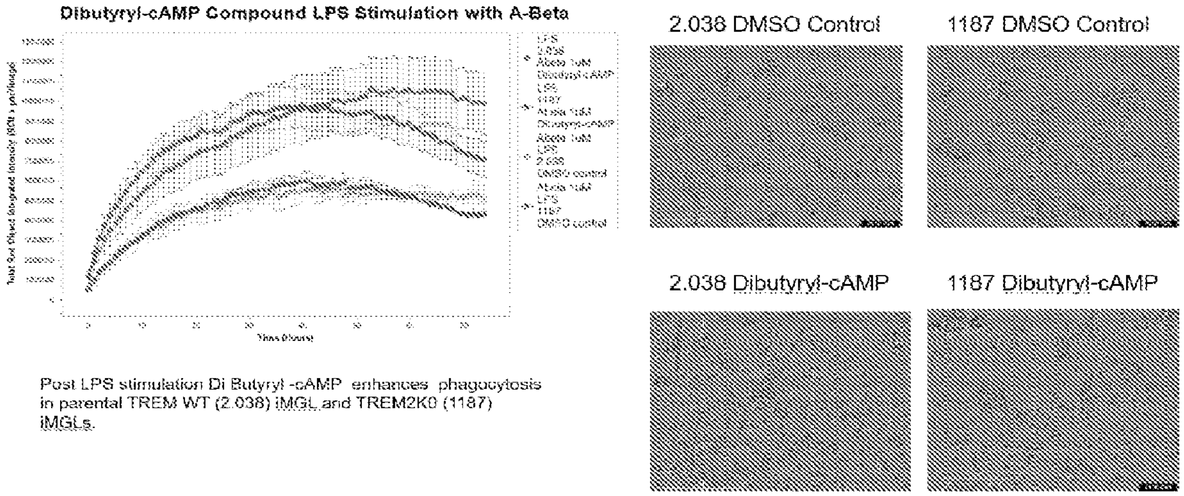
Post LPS stimulation Azeliragon inhibits phagocytosis in parental TREM WT (2.038) iMGLs, but it revealed an increase in phagocytosis in TREM2K0 (1187) iMGLs.

FIG. 40D



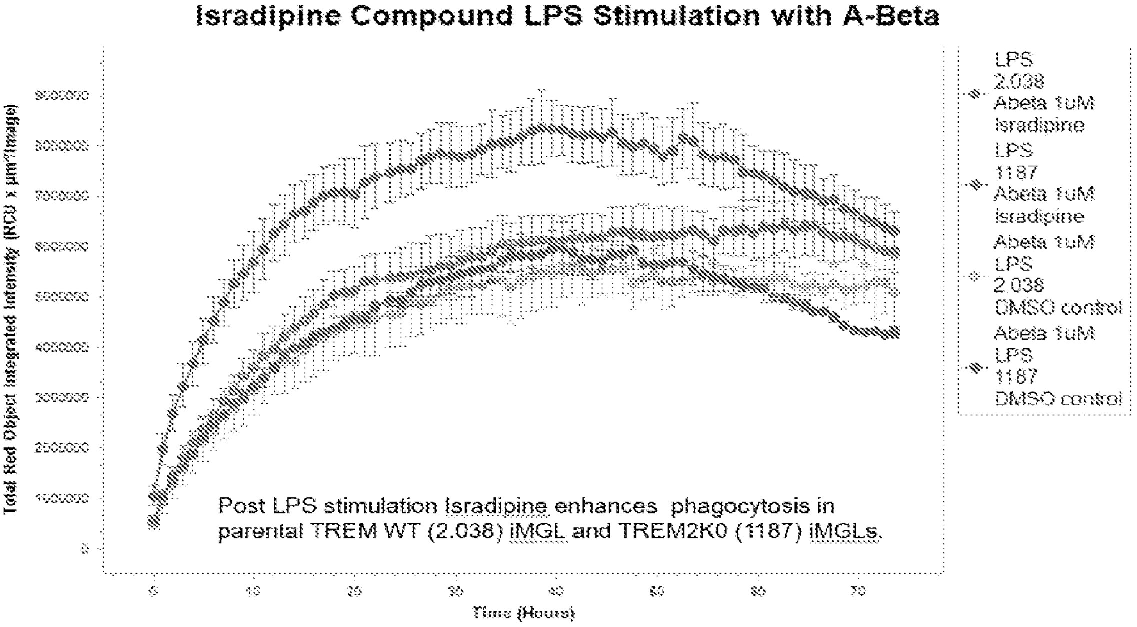
Post LPS stimulation Piceatannol enhances phagocytosis in parental TREM WT (2.038) iMGL and TREM2K0 (1187) iMGLs.

FIG. 41A



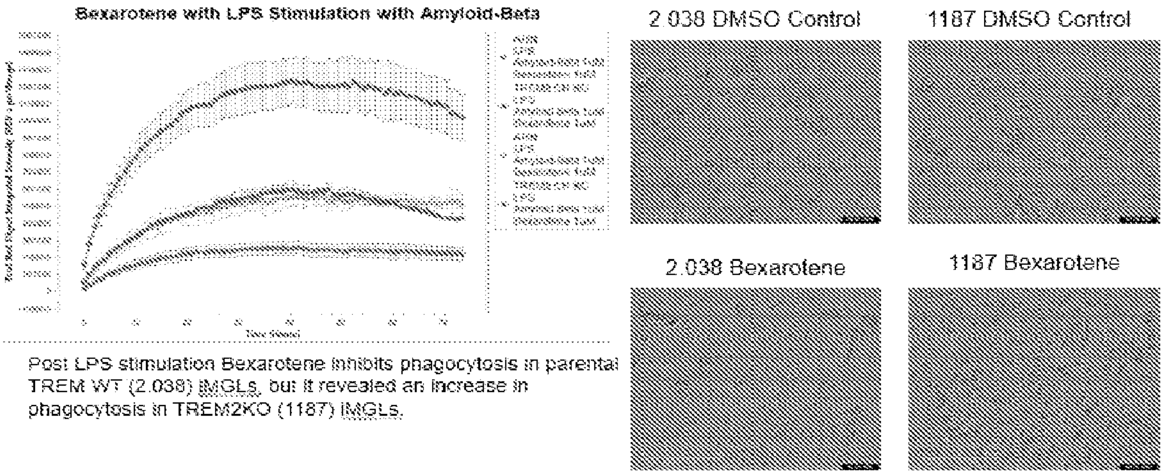
Post LPS stimulation Di Butyryl -cAMP enhances phagocytosis in parental TREM WT (2.038) iMGL and TREM2K0 (1187) iMGLs.

FIG. 41B



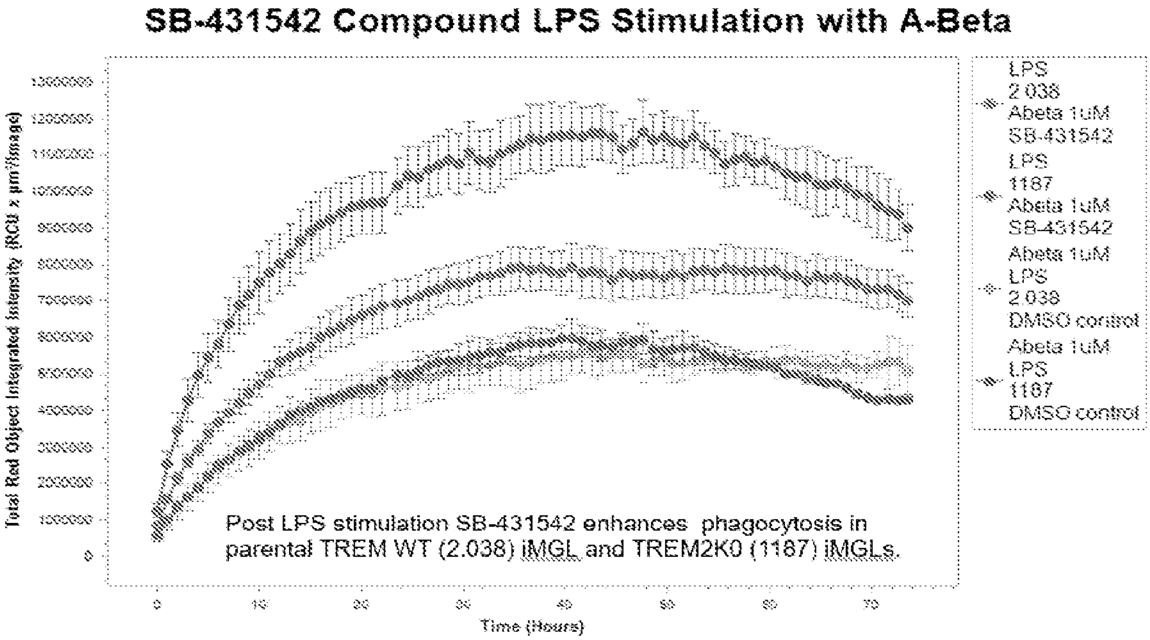
Post LPS stimulation Isradipine enhances phagocytosis in parental TREM WT (2.038) iMGL and TREM2K0 (1187) iMGLs.

FIG. 41C



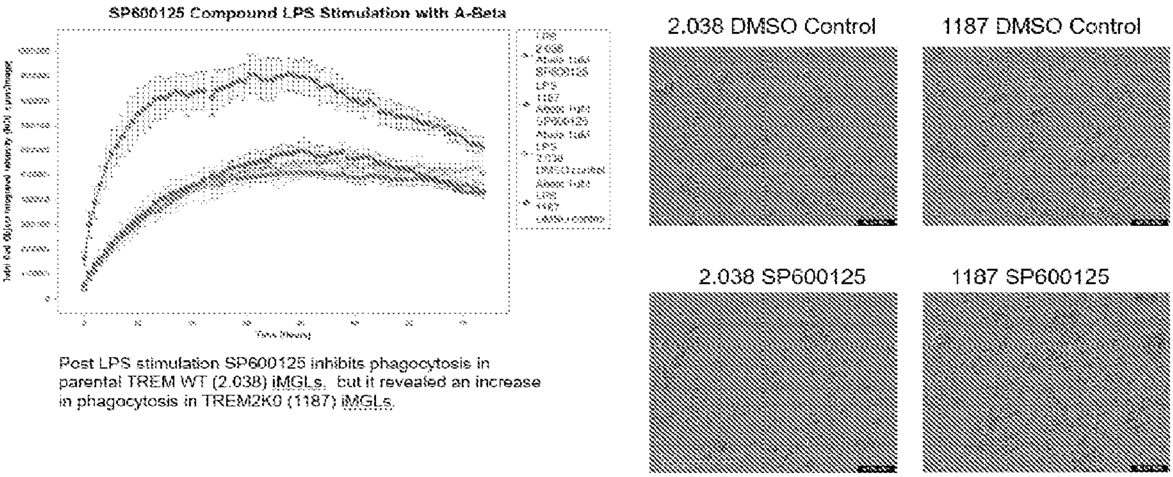
Post LPS stimulation Bexarotene inhibits phagocytosis in parental TREM WT (2.038) iMGLs, but it revealed an increase in phagocytosis in TREM2KO (1187) iMGLs.

FIG. 41D



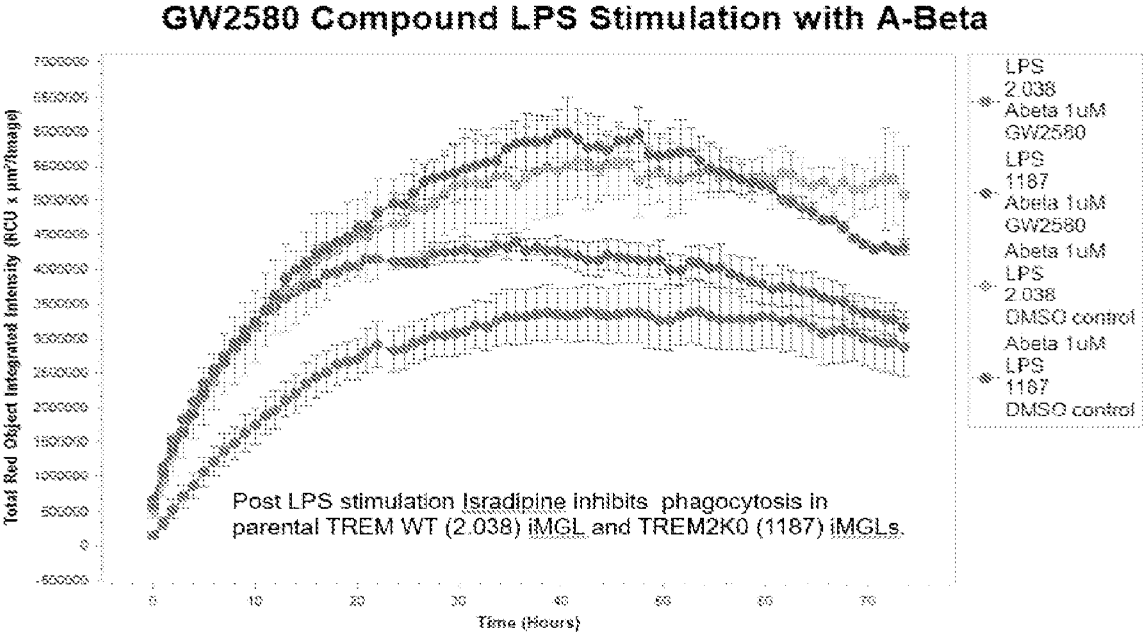
Post LPS stimulation SB-431542 enhances phagocytosis in parental TREM WT (2.038) iMGL and TREM2K0 (1187) iMGLs.

FIG. 41E



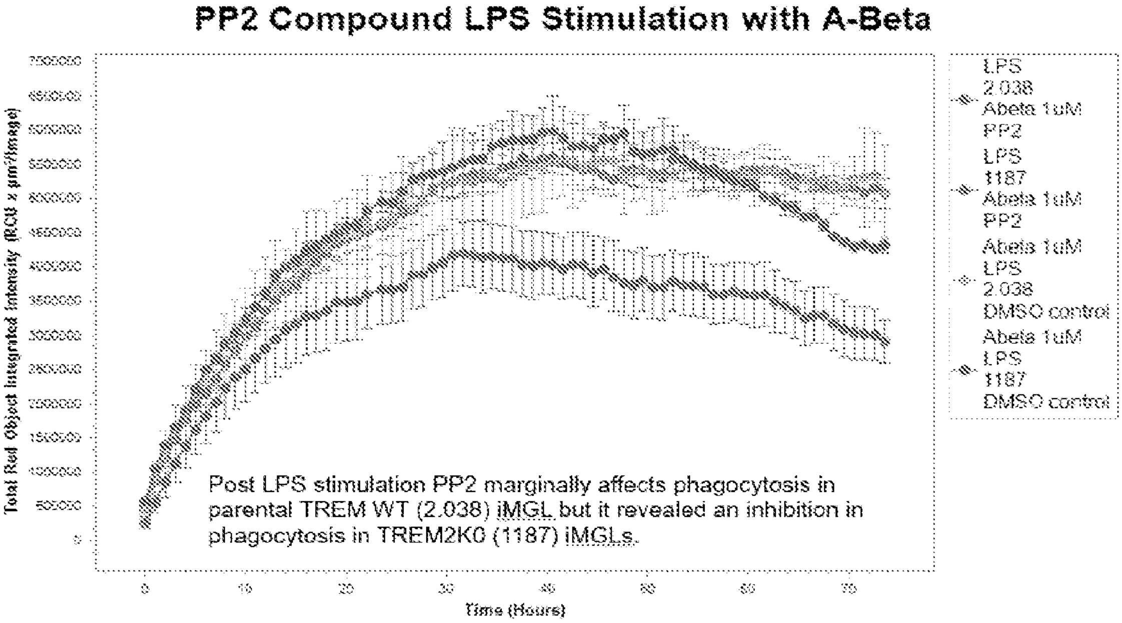
Post LPS stimulation SP600125 inhibits phagocytosis in parental TREM WT (2.038) iMGLs, but it revealed an increase in phagocytosis in TREM2K0 (1187) iMGLs.

FIG. 42A



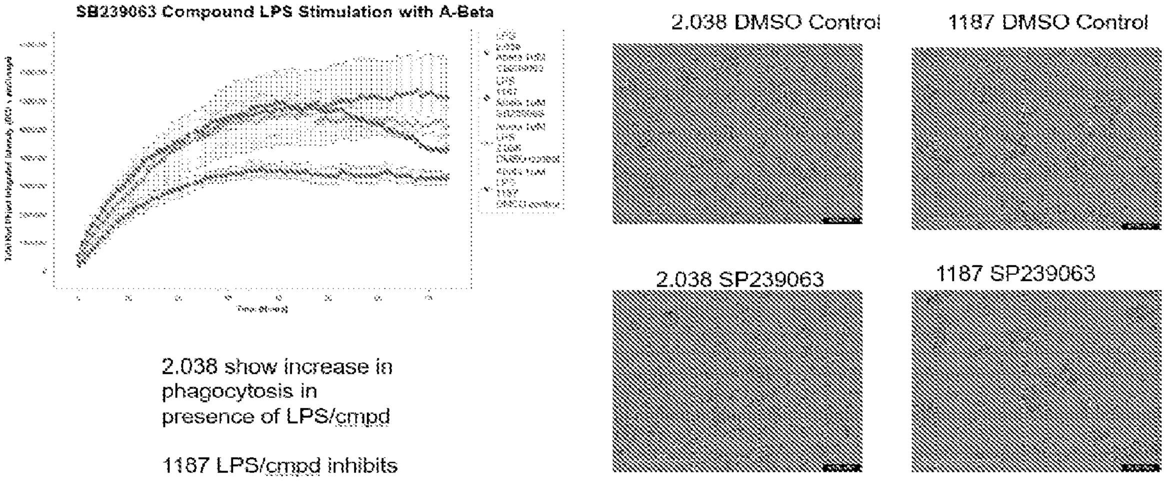
Post LPS stimulation Isradipine inhibits phagocytosis in parental TREM WT (2.038) iMGL and TREM2K0 (1187) iMGLs.

FIG. 42B


PP2 Compound LPS Stimulation with A-Beta

Post LPS stimulation PP2 marginally affects phagocytosis in parental TREM WT (2.038) iMGL but it revealed an inhibition in phagocytosis in TREM2KO (1187) iMGLs.

FIG. 42C


SB239063 Compound LPS Stimulation with A-Beta

2.038 DMSO Control

1187 DMSO Control

2.038 SP239063

1187 SP239063

2.038 show increase in phagocytosis in presence of LPS/cmpd

1187 LPS/cmpd inhibits

FIG. 42D

	Amyloid β Phagocytosis No LPS	Amyloid β Phagocytosis with LPS
Lucettine L41	Inhibits phagocytic function in AHN and TREM2HOKO iMGLs	Inhibits phagocytic function in AHN and TREM2HOKO iMGLs
GW501516	Inhibits phagocytic function in AHN and TREM2HOKO iMGLs	Increases phagocytic function in TREM2HOKO iMGLs. No difference in phagocytic function in AHN iMGLs
Piceatannol	Inhibits phagocytic function in AHN and TREM2HOKO iMGLs	Inhibits phagocytic function in AHN and TREM2HOKO iMGLs
Azeliragon	Increases phagocytic function in TREM2HOKO iMGLs and inhibits phagocytic function in AHN iMGLs	Increases phagocytic function in TREM2HOKO iMGLs and inhibits phagocytic function in AHN iMGLs
J147	Inhibits phagocytic function in both AHN iMGLs	Increases phagocytic function in AHN and TREM2HOKO iMGLs
Isradipine	Increases phagocytic function in TREM2HOKO iMGLs and inhibits phagocytic function in AHN iMGLs	Increases phagocytic function in AHN and TREM2HOKO iMGLs
Bexarotene	Inhibits phagocytic function in AHN and TREM2HOKO iMGLs	Increases phagocytic function in TREM2HOKO iMGLs and inhibits phagocytic function in AHN iMGLs
SB-431542	Increases phagocytic function in TREM2HOKO iMGLs and inhibits phagocytic function in AHN iMGLs	Increases phagocytic function in AHN and TREM2HOKO iMGLs
GW2580	Inhibits phagocytic function in AHN and TREM2HOKO iMGLs	Inhibits phagocytic function in AHN and TREM2HOKO iMGLs
SP600125	Increases phagocytic function in TREM2HOKO iMGLs and inhibits phagocytic function in AHN iMGLs	Increases phagocytic function in TREM2HOKO iMGLs and inhibits phagocytic function in AHN iMGLs
PP2	Inhibits phagocytic function in AHN and TREM2HOKO iMGLs	Inhibits phagocytic function in TREM2HOKO iMGLs and no difference in AHN iMGLs
SB239063	Inhibits phagocytic function in AHN and TREM2HOKO iMGLs	Marginal increase in phagocytic function in AHN iMGLs and inhibits phagocytic function in TREM2HOKO iMGLs

FIG. 43

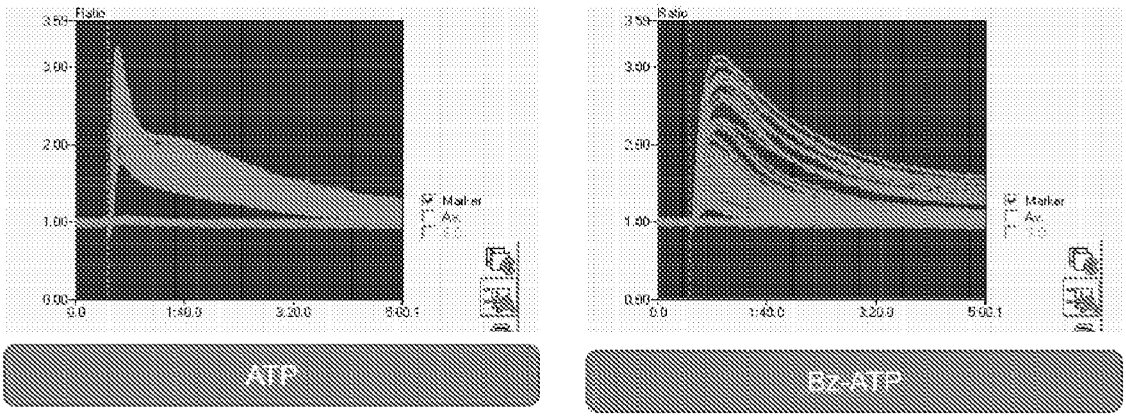


FIG. 44A

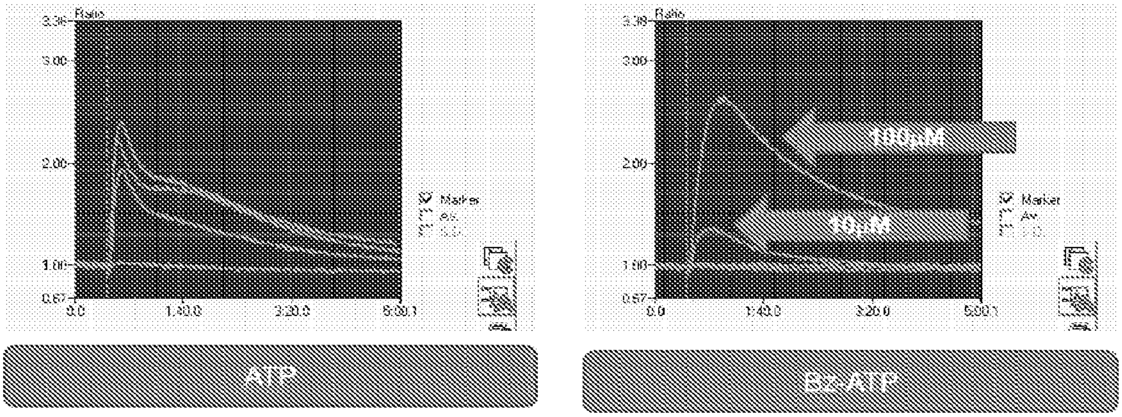


FIG. 44B

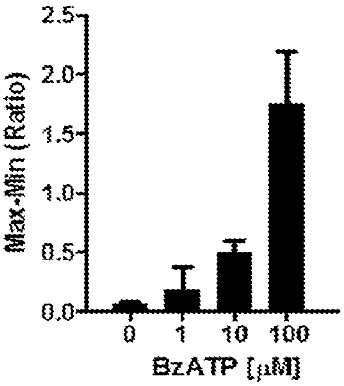


FIG. 44C

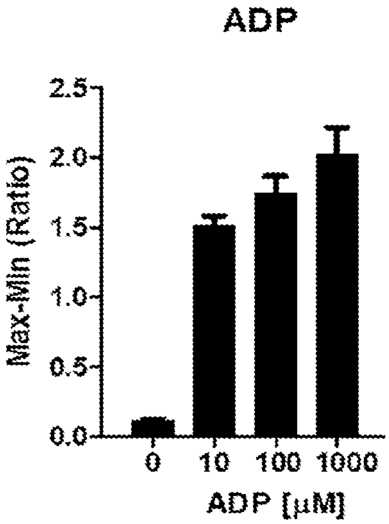


FIG. 44D

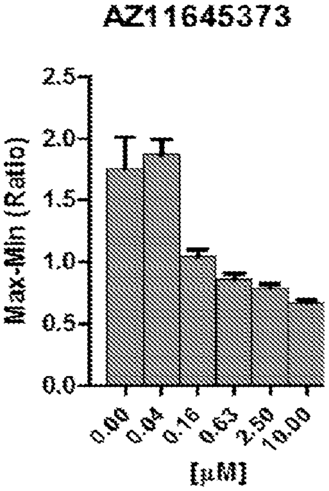


FIG. 44E

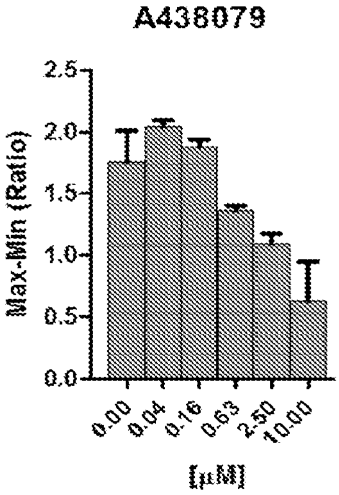


FIG. 44F

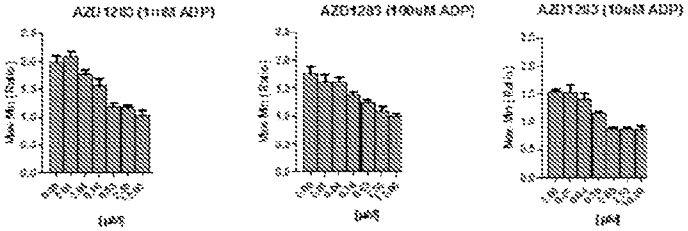


FIG. 44G

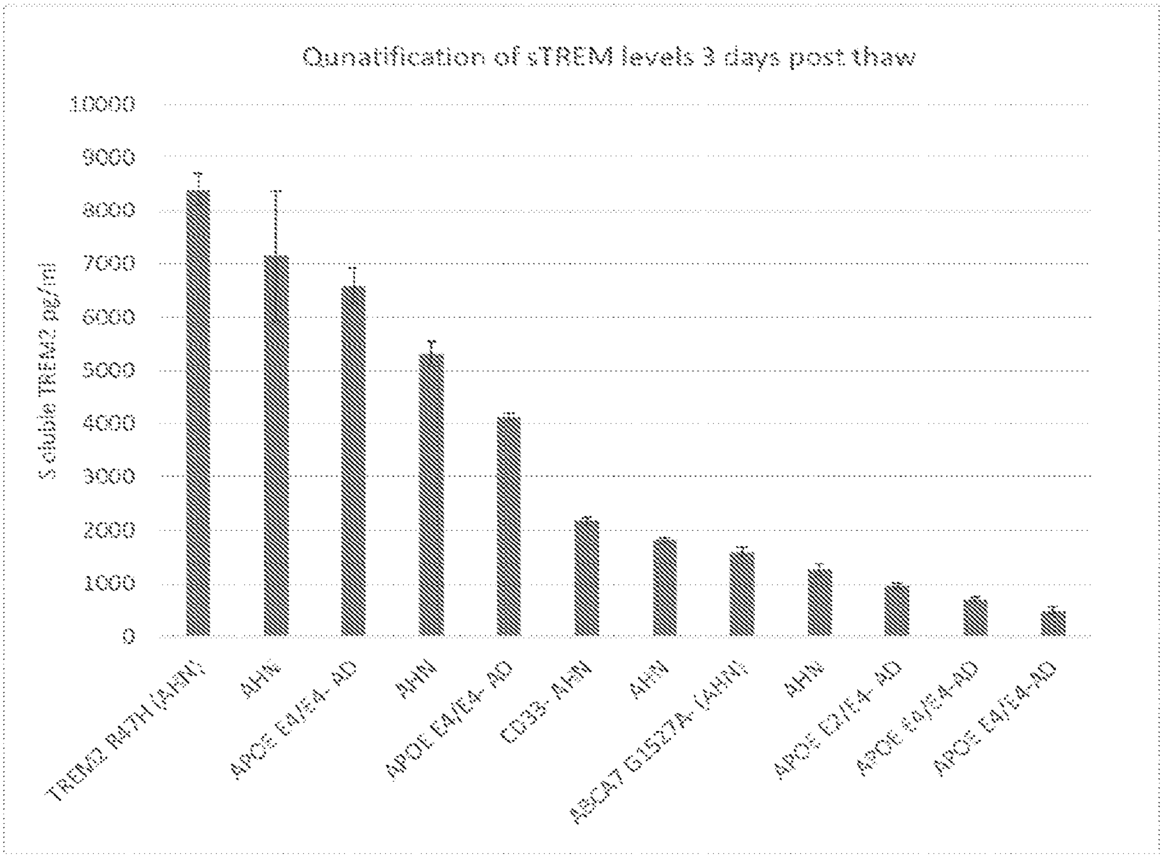

Qunatification of sTREM levels 3 days post thaw
Soluble TREM2 pg/ml
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
TREM2 R47H (AHN)
AHN
APOE E4/E4- AD
AHN
APOE E4/E4- AD
CD33- AHN
AHN
ABCA7 G1527A- (AHN)
AHN
APOE E2/E4- AD
APOE E4/E4-AD
APOE E4/E4-AD

FIG. 45A

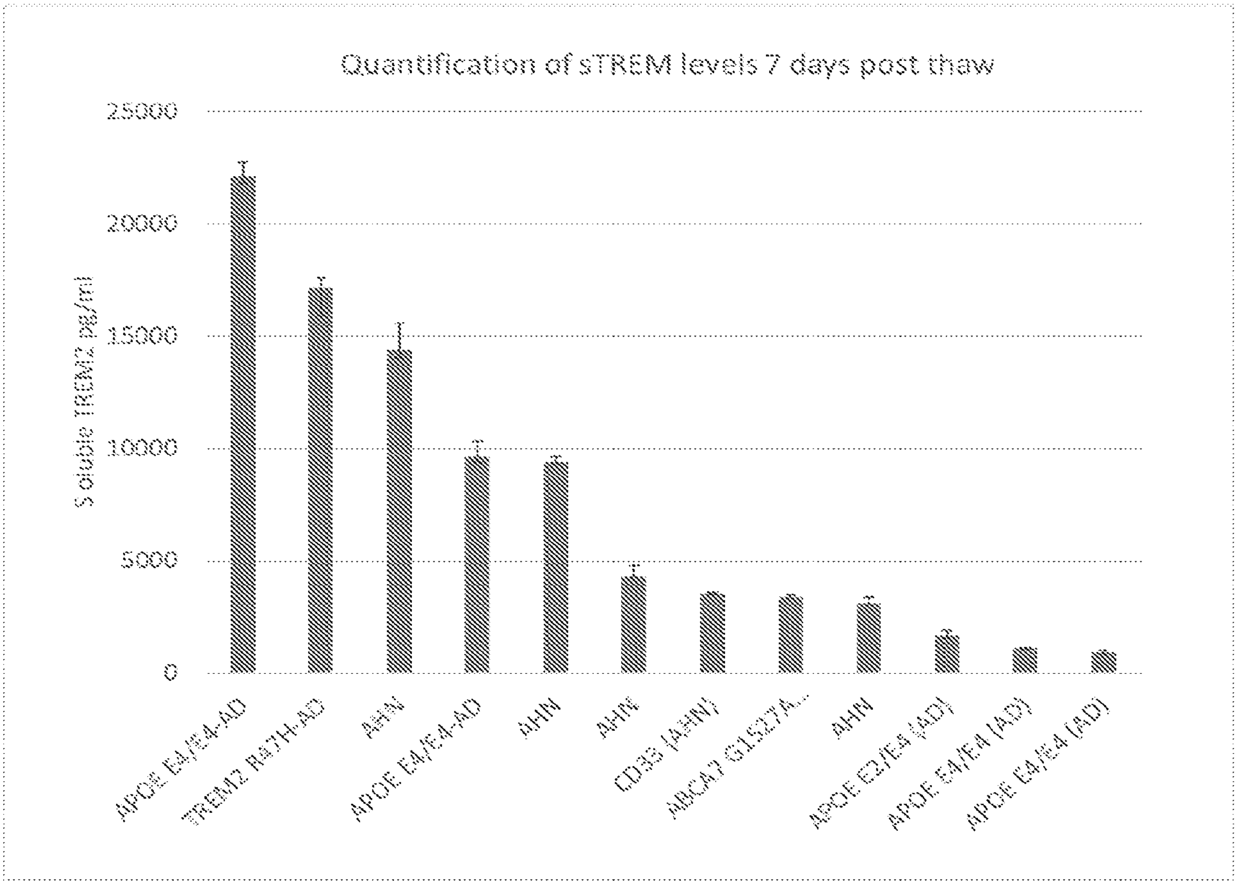

Quantification of sTREM levels 7 days post thaw
S oluble TREM2 pg/ml
25000
20000
15000
10000
5000
0
APOE E4/E4-AD
TREM2 R47H-AD
AHN
APOE E4/E4-AD
AHN
AHN
CD33 (AHN)
ABCA7 G1527A...
AHN
APOE E2/E4 (AD)
APOE E4/E4 (AD)
APOE E4/E4 (AD)

FIG. 45B

M1 Analytes Released from AHN and Disease Associated Microglia Lines Upon LPS Stimulation									
			Analyte (pg/mL)						
Genotype	Phenotype	Condition	IFN-gamma	IL-1 alpha / IL-1F1	IL-1 beta / IL-1F2	IL-6	IL-23	IL-27	TNF-alpha
APOE E2/E4	AD	Control	314	7	10	1	243	191	2
		LPS	321	10	14	565	381	131	79
TREM2 R47H	AHN	Control	473	9	7	1	352	249	3
		LPS	506	12	10	102	716	438	57
APOE E4/E4	AD	Control	74	5	18	0	440	108	1
		LPS	73	9	17	595	293	159	176
APOE E4/E4	AD	Control	342	7	3	1	223	0	2
		LPS	333	12	8	1529	553	29	165
ABCA7 G1527A	AHN	Control	297	6	5	0	182	6	1
		LPS	300	12	26	309	390	6	51
CD33	AHN	Control	453	10	8	1	380	130	3
		LPS	464	12	11	25	368	29	7
NA	AHN	Control	409	8	8	1	341	135	3
		LPS	375	11	12	276	547	175	138
NA	AHN	Control	530	11	8	1	368	6	4
		LPS	495	13	14	142	509	75	34
APOE E4/E4	AD	Control	364	12	6	0	276	0	2
		LPS	379	12	13	145	452	36	27
NA	AHN	Control	489	14	19	1	296	142	6
		LPS	468	16	17	355	423	54	58
APOE E4/E4	AD	Control	153	6	8	0	169	0	1
		LPS	179	14	15	2372	675	118	1021
NA	AHN	Control	222	5	4	0	232	0	1
		LPS	203	9	5	1076	827	22	470

FIG. 46A

M2 Analytes Released from AHN and Disease Associated Microglia Upon Stimulation with LPS					
			Analyte (pg/mL)		
Genotype	Phenotype	Condition	IL-1ra / IL-1F3	IL-10	IL-13
APOE E2/E4	AD	Control	1379	9	155
		LPS	1573	14	377
TREM2 R47H	AHN	Control	3045	12	273
		LPS	5339	73	442
APOE E4/E4	AD	Control	6535	7	251
		LPS	6489	8	432
APOE E4/E4	AD	Control	4072	9	86
		LPS	11184	147	486
ABCA7 G1527A	AHN	Control	3076	8	134
		LPS	3801	27	448
CD33	AHN	Control	4417	11	113
		LPS	5329	13	273
NA	AHN	Control	3504	12	216
		LPS	11649	119	739
NA	AHN	Control	2669	12	126
		LPS	4650	29	525
APOE E4/E4	AD	Control	5487	10	86
		LPS	6641	23	330
NA	AHN	Control	5388	12	49
		LPS	6955	27	414
APOE E4/E4	AD	Control	1274	5	227
		LPS	3988	67	844
NA	AHN	Control	3397	6	199
		LPS	3809	42	377

FIG. 46B

Interleukins Released from AHN and Disease Associated Microglia Upon Stimulation with LPS

			Analyte (pg/mL)								
Genotype	Phenotype	Condition	IL-1 alpha / IL-1F1	IL-1 beta / IL-1F2	IL-1ra / IL-1F3	IL-6	IL-8 / CXCL8	IL-10	IL-13	IL-23	IL-27
APOE E2/E4	AD	Control	7	10	1379	1	100	9	155	243	191
		LPS	10	14	1573	565	6766	14	377	381	131
TREM2 R47H	AHN	Control	9	7	3045	1	133	12	273	352	249
		LPS	12	10	5339	102	6354	73	442	716	438
APOE E4/E4	AD	Control	5	18	6535	0	153	7	251	440	108
		LPS	9	17	6489	595	6425	8	432	293	159
APOE E4/E4	AD	Control	7	3	4072	1	697	9	86	223	0
		LPS	12	8	11184	1529	7115	147	486	553	29
ABCA7 G1527A	AHN	Control	6	5	3076	0	64	8	134	182	6
		LPS	12	26	3801	309	6261	27	448	390	6
CD33	AHN	Control	10	8	4417	1	119	11	113	380	130
		LPS	12	11	5329	25	3775	13	273	368	29
NA	AHN	Control	8	8	3504	1	233	12	216	341	135
		LPS	11	12	11649	276	6870	119	739	547	175
NA	AHN	Control	11	8	2669	1	256	12	126	368	6
		LPS	13	14	4650	142	5834	29	525	509	75
APOE E4/E4	AD	Control	12	6	5487	0	118	10	86	276	0
		LPS	12	13	6641	145	5210	23	330	452	36
NA	AHN	Control	14	19	5388	1	385	12	49	296	142
		LPS	16	17	6955	355	6736	27	414	423	54
APOE E4/E4	AD	Control	6	8	1274	0	250	5	227	169	0
		LPS	14	15	3988	2372	7019	67	844	675	118
NA	AHN	Control	5	4	3397	0	157	6	199	232	0
		LPS	9	5	3809	1076	6801	42	377	827	22

FIG. 46C

Chemokines Released from AHN and Disease Associated Microglia Upon Stimulation with LPS

			Analyte (pg/mL)									
Genotype	Phenotype	Condition	CCL2 / JE / MCP-1	CCL3 / MIP-1 alpha	CCL4 / MIP-1 beta	CCL20 / MIP-3 alpha	CCL22 / MDC	CCL24 / Eotaxin-2 / MPIF-2	CX3CL1 / Fractalkine	CXCL1 / GRO alpha / KC / CINC-1	CXCL11 / I-TAC	IL-8 / CXCL8
APOE E2/E4	AD	Control	175	200	245	22	2001	60	1294	0	75	100
		LPS	1352	3436	5230	2476	5983	71	1562	4226	37	6756
TREM2 R47H	AHN	Control	108	264	314	49	1661	100	1944	88	0	133
		LPS	2631	3526	4445	3698	4916	106	2429	2966	227	6354
APOE E4/E4	AD	Control	165	192	133	19	11065	43	694	0	0	153
		LPS	1257	13050	8657	2977	14416	50	638	3111	64	6425
APOE E4/E4	AD	Control	1366	221	332	57	4571	376	1321	315	0	697
		LPS	15003	18393	22450	5115	7164	343	1544	9482	674	7115
ABCA7 G1527A	AHN	Control	248	184	218	21	2395	65	1084	0	0	64
		LPS	2680	3985	8483	3673	9322	66	1512	1728	27	6251
CD33	AHN	Control	447	264	289	31	10447	325	1786	190	104	119
		LPS	543	1367	3689	1370	26113	288	2087	449	23	3775
NA	AHN	Control	277	232	359	70	977	149	1721	70	295	233
		LPS	3021	9971	21082	5092	2317	135	1920	5060	24	6870
NA	AHN	Control	620	234	308	25	4704	682	2016	45	0	256
		LPS	2164	4237	9507	2687	16215	558	2134	1756	127	5834
APOE E4/E4	AD	Control	175	227	256	22	13203	54	1525	0	178	118
		LPS	335	1907	3014	3626	35837	61	1699	1421	9	5210
NA	AHN	Control	787	267	332	35	43851	589	2112	51	0	385
		LPS	2202	6787	7460	2373	48029	504	2148	2861	237	6736
APOE E4/E4	AD	Control	1112	134	218	17	1018	86	754	115	0	250
		LPS	7948	32688	24775	6271	5057	91	1177	10492	267	7019
NA	AHN	Control	109	180	263	31	1281	171	1056	207	0	157
		LPS	3415	21786	8967	4011	1336	157	1177	4401	178	6801

FIG. 46D

Other Analytes Released from AHN and Disease Associated Microglia Upon Stimulation with LPS								
			Analyte (pg/mL)					
Genotype	Phenotype	Condition	B7-H1 / PD-L1	CD163	Chitinase 3-like 1	MMP-9	MMP-12	alpha-Synuclein
APOE E2/E4	AD	Control	58	10269	2984	177228	45	119
		LPS	112	18589	5525	170700	43	100
TREM2 R47H	AHN	Control	118	12574	23451	315682	30	125
		LPS	637	23227	52451	285808	39	112
APOE E4/E4	AD	Control	347	13046	6864	35744	44	239
		LPS	82	21143	9316	29152	24	256
APOE E4/E4	AD	Control	53	11608	70563	197729	31	104
		LPS	136	32713	140563	179716	75	99
ABCA7 G1527A	AHN	Control	35	10604	13033	156685	14	125
		LPS	125	18711	24055	160657	17	92
CD33	AHN	Control	179	14553	18284	300500	48	133
		LPS	97	15753	21225	299275	14	105
NA	AHN	Control	205	10847	24612	263545	27	174
		LPS	153	29038	48662	200213	39	87
NA	AHN	Control	109	12254	12535	355246	7	144
		LPS	106	20217	26394	320025	0	110
APOE E4/E4	AD	Control	88	13043	10784	233550	25	122
		LPS	97	20340	22846	207336	25	102
NA	AHN	Control	50	16738	5724	322137	51	203
		LPS	167	22026	11145	296418	7	153
APOE E4/E4	AD	Control	26	10269	16464	80299	147	210
		LPS	71	37162	23244	71914	295	187
NA	AHN	Control	59	9574	21829	132360	24	117
		LPS	44	23234	25735	110216	21	99

FIG. 46E

M1 Analytes Released from AHN and Disease Associated Microglia Upon Stimulation with IL-4 + dBu-cAMP

			Analyte (pg/mL)			
Genotype	Phenotype	Condition	IFN-gamma	IL-12 p70	IL-23	IL-27
APOE E2/E4	AD	Control	314	15	243	191
		IL-4 + dBu-cAMP	392	264	502	502
TREM2 R47H	AHN	Control	473	24	352	249
		IL-4 + dBu-cAMP	462	19	316	142
APOE E4/E4	AD	Control	74	20	440	108
		IL-4 + dBu-cAMP	77	4	112	36
APOE E4/E4	AD	Control	342	5	223	0
		IL-4 + dBu-cAMP	328	17	475	60
ABCA7 G1527A	AHN	Control	297	9	182	6
		IL-4 + dBu-cAMP	282	12	196	6
CD33	AHN	Control	453	20	380	130
		IL-4 + dBu-cAMP	493	20	322	0
NA	AHN	Control	409	58	341	135
		IL-4 + dBu-cAMP	412	215	505	328
NA	AHN	Control	530	20	368	6
		IL-4 + dBu-cAMP	462	17	309	82
APOE E4/E4	AD	Control	364	15	276	0
		IL-4 + dBu-cAMP	402	17	213	64
NA	AHN	Control	489	22	296	142
		IL-4 + dBu-cAMP	506	49	374	170
APOE E4/E4	AD	Control	153	10	169	0
		IL-4 + dBu-cAMP	137	9	134	0
NA	AHN	Control	222	10	232	0
		IL-4 + dBu-cAMP	219	14	169	66

FIG. 46F

M2 Analytes Released from AHN and Disease Associated Microglia Upon Stimulation with IL-4 + dBu-cAMP					
			Analyte (pg/mL)		
Genotype	Phenotype	Condition	IL-1ra / IL-1F3	IL-10	IL-13
APOE E2/E4	AD	Control	1379	9	155
		IL-4 + dBu-cAMP	2435	17	510
TREM2 R47H	AHN	Control	3045	12	273
		IL-4 + dBu-cAMP	5282	12	228
APOE E4/E4	AD	Control	6535	7	251
		IL-4 + dBu-cAMP	6295	4	49
APOE E4/E4	AD	Control	4072	9	86
		IL-4 + dBu-cAMP	10559	11	49
ABCA7 G1527A	AHN	Control	3076	8	134
		IL-4 + dBu-cAMP	5332	8	86
CD33	AHN	Control	4417	11	113
		IL-4 + dBu-cAMP	6214	13	162
NA	AHN	Control	3504	12	216
		IL-4 + dBu-cAMP	6995	15	411
NA	AHN	Control	2669	12	126
		IL-4 + dBu-cAMP	3905	11	134
APOE E4/E4	AD	Control	5487	10	86
		IL-4 + dBu-cAMP	6564	9	134
NA	AHN	Control	5388	12	49
		IL-4 + dBu-cAMP	5880	14	208
APOE E4/E4	AD	Control	1274	5	227
		IL-4 + dBu-cAMP	1898	4	171
NA	AHN	Control	3397	6	199
		IL-4 + dBu-cAMP	4712	6	214

FIG. 46G

Interleukins Released from AHN and Disease Associated Microglia Upon Stimulation with IL-4 + dBu-cAMP									
			Analyte (pg/mL)						
Genotype	Phenotype	Condition	IL-1ra / IL-1F3	IL-8 / CXCL8	IL-10	IL-12 p70	IL-13	IL-23	IL-27
APOE E2/E4	AD	Control	1379	100	9	15	155	243	191
		IL-4 + dBu-cAMP	2435	144	17	264	510	502	502
TREM2 R47H	AHN	Control	3045	133	12	24	273	352	249
		IL-4 + dBu-cAMP	5282	96	12	19	228	316	142
APOE E4/E4	AD	Control	6535	153	7	20	251	440	108
		IL-4 + dBu-cAMP	6295	168	4	4	49	112	36
APOE E4/E4	AD	Control	4072	697	9	5	86	223	0
		IL-4 + dBu-cAMP	10559	568	11	17	49	475	60
ABCA7 G1527A	AHN	Control	3076	64	8	9	134	182	6
		IL-4 + dBu-cAMP	5332	56	8	12	86	196	6
CD33	AHN	Control	4417	119	11	20	113	380	130
		IL-4 + dBu-cAMP	6214	128	13	20	162	322	0
NA	AHN	Control	3504	233	12	58	216	341	135
		IL-4 + dBu-cAMP	6995	159	15	215	411	505	328
NA	AHN	Control	2669	256	12	20	126	368	6
		IL-4 + dBu-cAMP	3905	193	11	17	134	309	82
APOE E4/E4	AD	Control	5487	118	10	15	86	276	0
		IL-4 + dBu-cAMP	6564	132	9	17	134	213	64
NA	AHN	Control	5388	385	12	22	49	296	142
		IL-4 + dBu-cAMP	5880	371	14	49	208	374	170
APOE E4/E4	AD	Control	1274	250	5	10	227	169	0
		IL-4 + dBu-cAMP	1898	206	4	9	171	134	0
NA	AHN	Control	3397	157	6	10	199	232	0
		IL-4 + dBu-cAMP	4712	188	6	14	214	169	66

FIG. 46H

Chemokines Released from AHN and Disease Associated Microglia Upon Stimulation with IL-4 + dBu-cAMP

			Analyte (pg/mL)											
Genotype	Phenotype	Condition	CCL2 / JE / MCP-1	CCL3 / MIP-1 alpha	CCL4 / MIP-1 beta	CCL13 / MCP-4	CCL17 / TARC	CCL22 / MDC	CCL24 / Eotaxin-2 /	CCL26 / Eotaxin-3	CX3CL1 / Fractalkine	CXCL1 / GRO alpha / KC /	CXCL11 / I-TAC	IL-8 / CXCL8
APOE E2/E4	AD	Control	175	200	245	5	65	2001	60	10	1294	0	75	100
		IL-4 + dBu-cAMP	225	264	308	257	203	2805	139	21	1772	209	5	144
TREM2 R47H	AHN	Control	108	264	314	6	126	1661	100	12	1944	88	0	133
		IL-4 + dBu-cAMP	134	283	338	1631	2003	2763	375	166	2016	441	0	96
APOE E4/E4	AD	Control	165	192	133	8	7	11065	43	8	694	0	0	153
		IL-4 + dBu-cAMP	148	158	120	183	40	9822	65	14	621	127	7	168
APOE E4/E4	AD	Control	1366	221	332	14	40	4571	376	9	1321	315	0	697
		IL-4 + dBu-cAMP	1552	268	283	868	393	8114	1215	283	1647	361	193	568
ABCA7 G1527A	AHN	Control	248	184	218	5	40	2395	65	10	1084	0	0	64
		IL-4 + dBu-cAMP	268	207	218	1036	462	3386	233	71	1138	59	98	56
CD33	AHN	Control	447	264	289	5	82	10447	325	10	1786	190	104	119
		IL-4 + dBu-cAMP	432	264	320	1267	866	10319	413	50	1871	255	0	128
NA	AHN	Control	277	232	359	4	96	977	149	12	1721	70	295	233
		IL-4 + dBu-cAMP	308	286	342	1271	530	1274	701	179	2057	131	79	159
NA	AHN	Control	620	234	308	12	112	4704	682	11	2016	45	0	256
		IL-4 + dBu-cAMP	634	261	320	455	326	6425	784	22	1919	58	96	193
APOE E4/E4	AD	Control	175	227	256	12	56	13203	54	9	1525	0	178	118
		IL-4 + dBu-cAMP	169	227	263	405	1167	19089	181	19	1474	0	0	132
NA	AHN	Control	787	267	332	16	112	43851	589	13	2112	51	0	385
		IL-4 + dBu-cAMP	990	291	355	726	851	44441	757	65	2325	93	1	371
APOE E4/E4	AD	Control	1112	134	218	7	40	1018	86	9	754	115	0	250
		IL-4 + dBu-cAMP	964	139	193	847	224	1151	230	66	725	0	13	206
NA	AHN	Control	109	180	263	8	65	1281	171	9	1056	207	0	157
		IL-4 + dBu-cAMP	141	199	252	557	82	1456	358	47	949	205	0	188

FIG. 46I

Other Analytes Released from AHN and Disease Associated Microglia Upon Stimulation with IL-4 + dBu-cAMP								
			Analyte (pg/mL)					
Genotype	Phenotype	Condition	B7-H1 / PD-L1	CD163	Chitinase 3-like 1	MMP-9	MMP-12	alpha-Synuclein
APOE E2/E4	AD	Control	58	10269	2984	177228	45	119
		IL-4 + dBu-cAMP	678	13799	3728	208780	85	171
TREM2 R47H	AHN	Control	118	12574	23451	315682	30	125
		IL-4 + dBu-cAMP	91	11211	24034	309859	41	101
APOE E4/E4	AD	Control	347	13046	6864	35744	44	239
		IL-4 + dBu-cAMP	64	11593	6518	35541	17	217
APOE E4/E4	AD	Control	53	11608	70563	197729	31	104
		IL-4 + dBu-cAMP	268	18907	76950	176195	57	111
ABCA7 G1527A	AHN	Control	35	10604	13033	156685	14	125
		IL-4 + dBu-cAMP	61	10269	12213	152782	0	95
CD33	AHN	Control	179	14553	18284	300500	48	133
		IL-4 + dBu-cAMP	77	13180	17085	323735	65	115
NA	AHN	Control	205	10847	24612	263545	27	174
		IL-4 + dBu-cAMP	517	12915	25043	232950	59	131
NA	AHN	Control	109	12254	12535	355246	7	144
		IL-4 + dBu-cAMP	64	14113	12283	332798	30	139
APOE E4/E4	AD	Control	88	13043	10784	233550	25	122
		IL-4 + dBu-cAMP	52	13799	11197	221060	65	132
NA	AHN	Control	50	16738	5724	322137	51	203
		IL-4 + dBu-cAMP	192	17885	6193	345169	74	211
APOE E4/E4	AD	Control	26	10269	16464	80299	147	210
		IL-4 + dBu-cAMP	29	10947	13897	72061	152	179
NA	AHN	Control	59	9574	21829	132360	24	117
		IL-4 + dBu-cAMP	35	9926	23114	130927	38	136

FIG. 46J

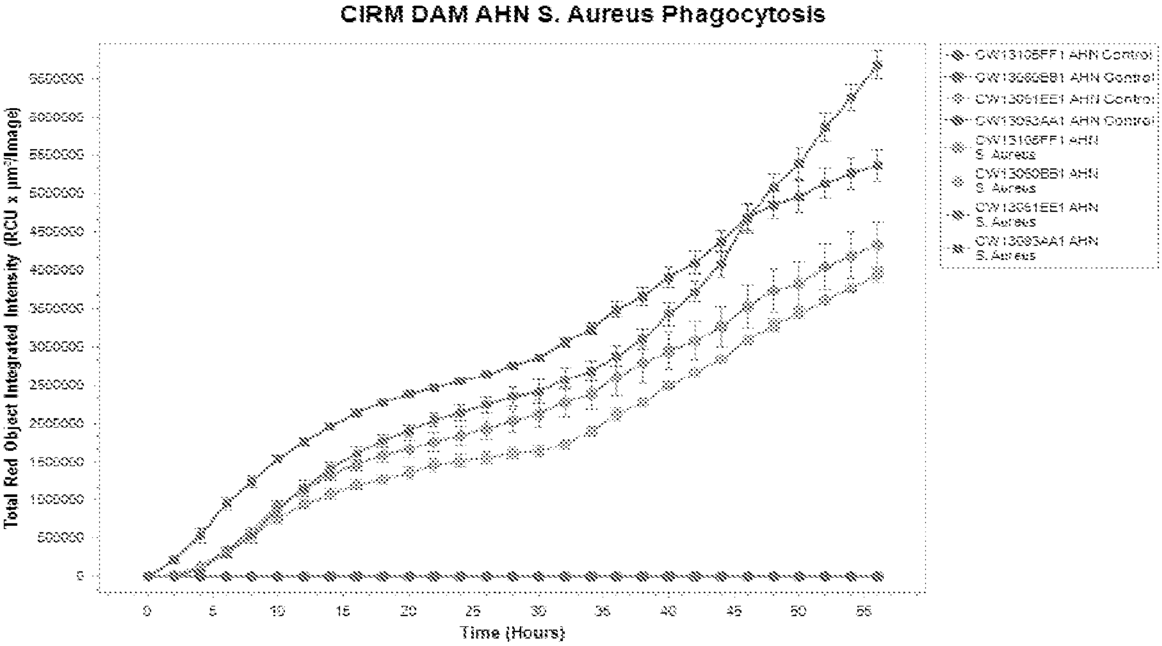

CIRM DAM AHN S. Aureus Phagocytosis
Total Red Object Integrated Intensity (RCU x µm²/Image)
6500000
6000000
5500000
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
0
5
10
15
20
25
30
35
40
45
50
55
Time (Hours)
CW13105FF1 AHN Control
CW13060BB1 AHN Control
CW13061EE1 AHN Control
CW13093AA1 AHN Control
CW13105FF1 AHN S. Aureus
CW13060BB1 AHN S. Aureus
CW13061EE1 AHN S. Aureus
CW13093AA1 AHN S. Aureus

FIG. 47A

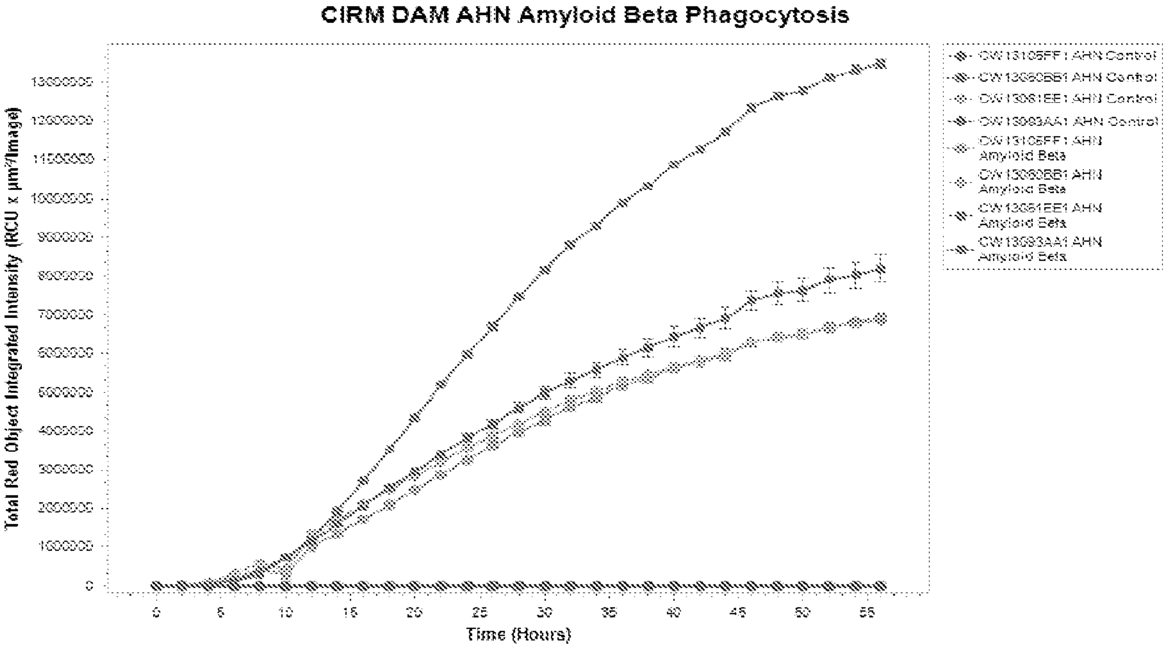

CIRM DAM AHN Amyloid Beta Phagocytosis
Total Red Object Integrated Intensity (RCU x µm²/Image)
13000000
12000000
11000000
10000000
9000000
8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
0
5
10
15
20
25
30
35
40
45
50
55
Time (Hours)
CW13105FF1 AHN Control
CW13060BB1 AHN Control
CW13061EE1 AHN Control
CW13093AA1 AHN Control
CW13105FF1 AHN Amyloid Beta
CW13060BB1 AHN Amyloid Beta
CW13061EE1 AHN Amyloid Beta
CW13093AA1 AHN Amyloid Beta

FIG. 47B

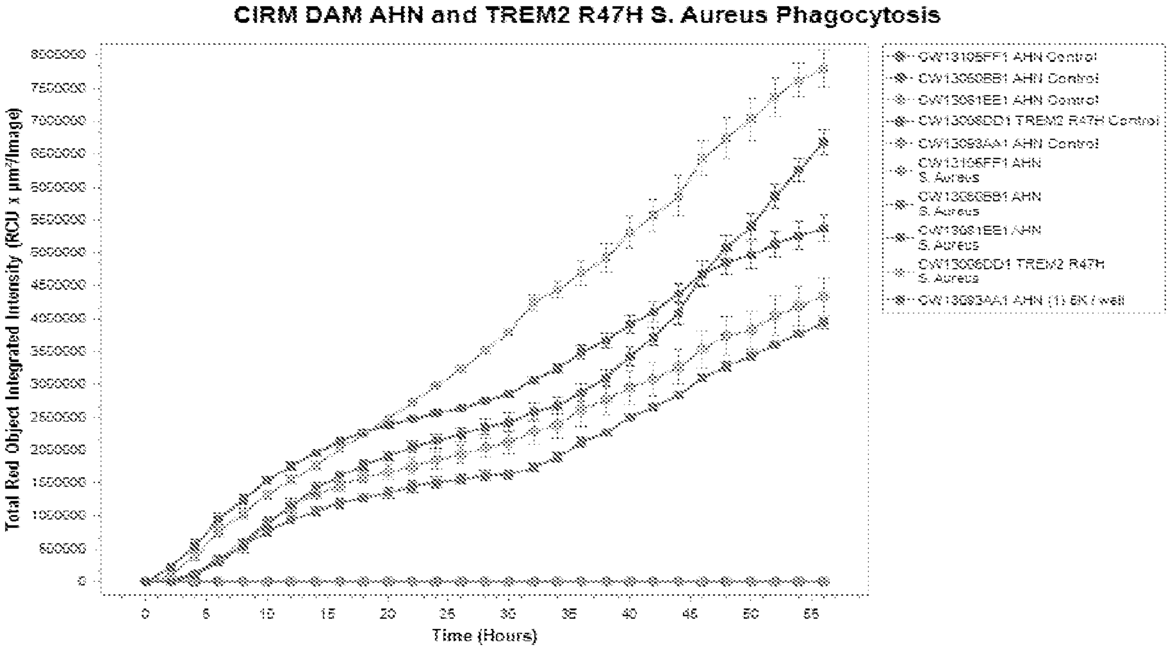

CIRM DAM AHN and TREM2 R47H S. Aureus Phagocytosis
Total Red Object Integrated Intensity (RCU x µm²/Image)
8000000
7500000
7000000
6500000
6000000
5500000
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
0
5
10
15
20
25
30
35
40
45
50
55
Time (Hours)
CW13105FF1 AHN Control
CW13060BB1 AHN Control
CW13061EE1 AHN Control
CW13006DD1 TREM2 R47H Control
CW13093AA1 AHN Control
CW13105FF1 AHN S. Aureus
CW13060BB1 AHN S. Aureus
CW13061EE1 AHN S. Aureus
CW13006DD1 TREM2 R47H S. Aureus
CW13093AA1 AHN (1) 6K / well

FIG. 47C

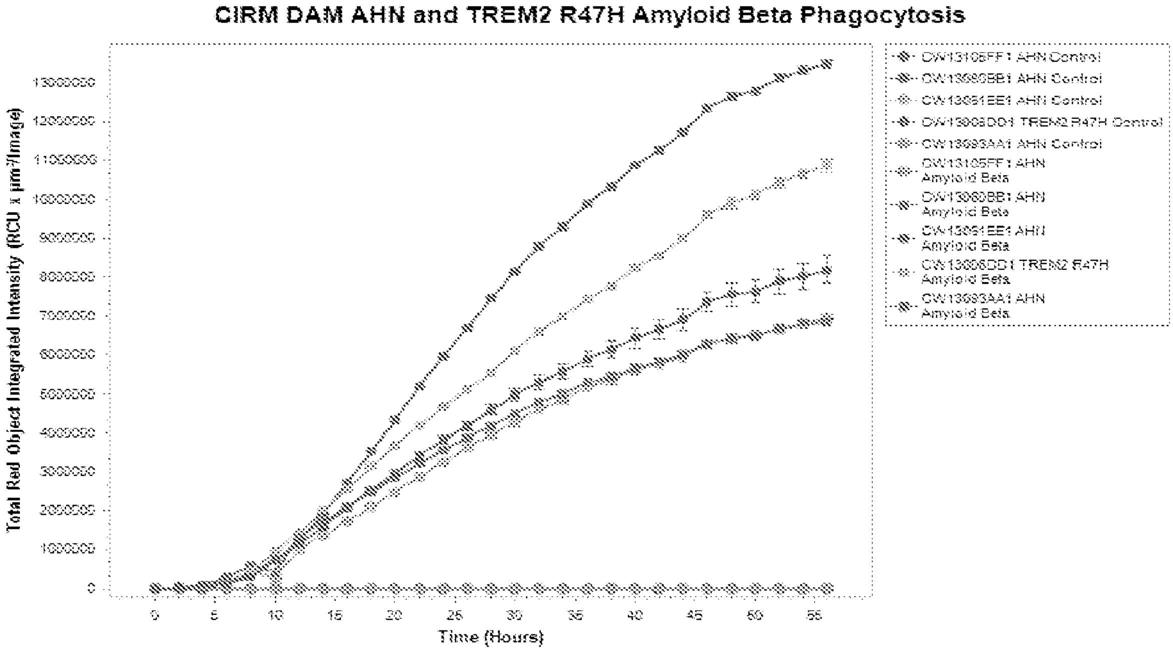

CIRM DAM AHN and TREM2 R47H Amyloid Beta Phagocytosis
Total Red Object Integrated Intensity (RCU x µm²/Image)
13000000
12000000
11000000
10000000
9000000
8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
0
5
10
15
20
25
30
35
40
45
50
55
Time (Hours)
CW13105FF1 AHN Control
CW13080BB1 AHN Control
CW13091EE1 AHN Control
CW13006DD1 TREM2 R47H Control
CW13093AA1 AHN Control
CW13105FF1 AHN Amyloid Beta
CW13080BB1 AHN Amyloid Beta
CW13091EE1 AHN Amyloid Beta
CW13006DD1 TREM2 R47H Amyloid Beta
CW13093AA1 AHN Amyloid Beta

FIG. 47D

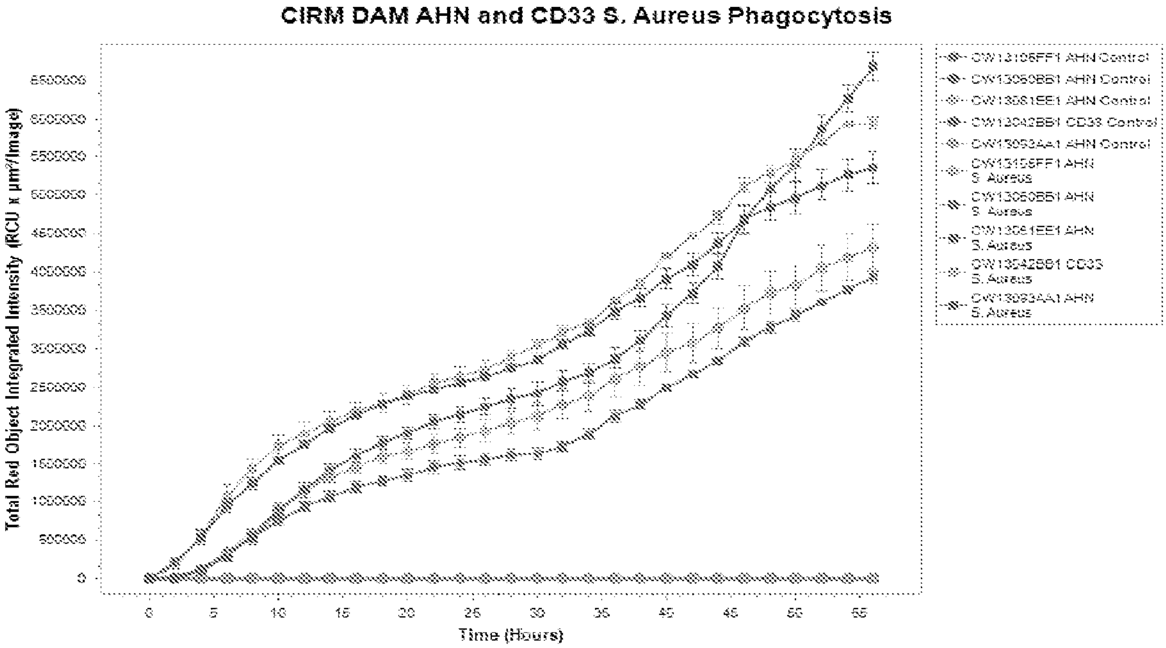

CIRM DAM AHN and CD33 S. Aureus Phagocytosis
Total Red Object Integrated Intensity (RCU x µm²/Image)
6500000
6000000
5500000
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
0
5
10
15
20
25
30
35
40
45
50
55
Time (Hours)
CW13105FF1 AHN Control
CW13060BB1 AHN Control
CW13081EE1 AHN Control
CW13042BB1 CD33 Control
CW13093AA1 AHN Control
CW13155FF1 AHN S. Aureus
CW13060BB1 AHN S. Aureus
CW13081EE1 AHN S. Aureus
CW13542BB1 CD33 S. Aureus
CW13093AA1 AHN S. Aureus

FIG. 47E

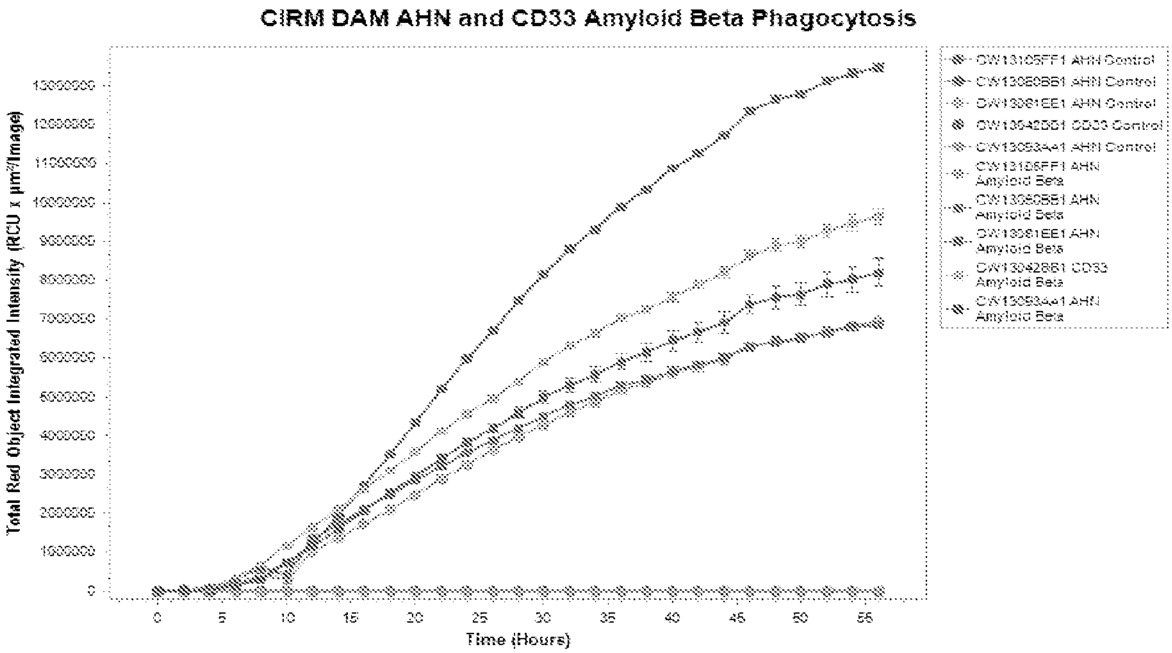

CIRM DAM AHN and CD33 Amyloid Beta Phagocytosis
Total Red Object Integrated Intensity (RCU x µm²/Image)
13000000
12000000
11000000
10000000
9000000
8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
0
5
10
15
20
25
30
35
40
45
50
55
Time (Hours)
CW13106FF1 AHN Control
CW13060BB1 AHN Control
CW13081EE1 AHN Control
CW13042BB1 CD33 Control
CW13093AA1 AHN Control
CW13106FF1 AHN Amyloid Beta
CW13060BB1 AHN Amyloid Beta
CW13081EE1 AHN Amyloid Beta
CW13042BB1 CD33 Amyloid Beta
CW13093AA1 AHN Amyloid Beta

FIG. 47F

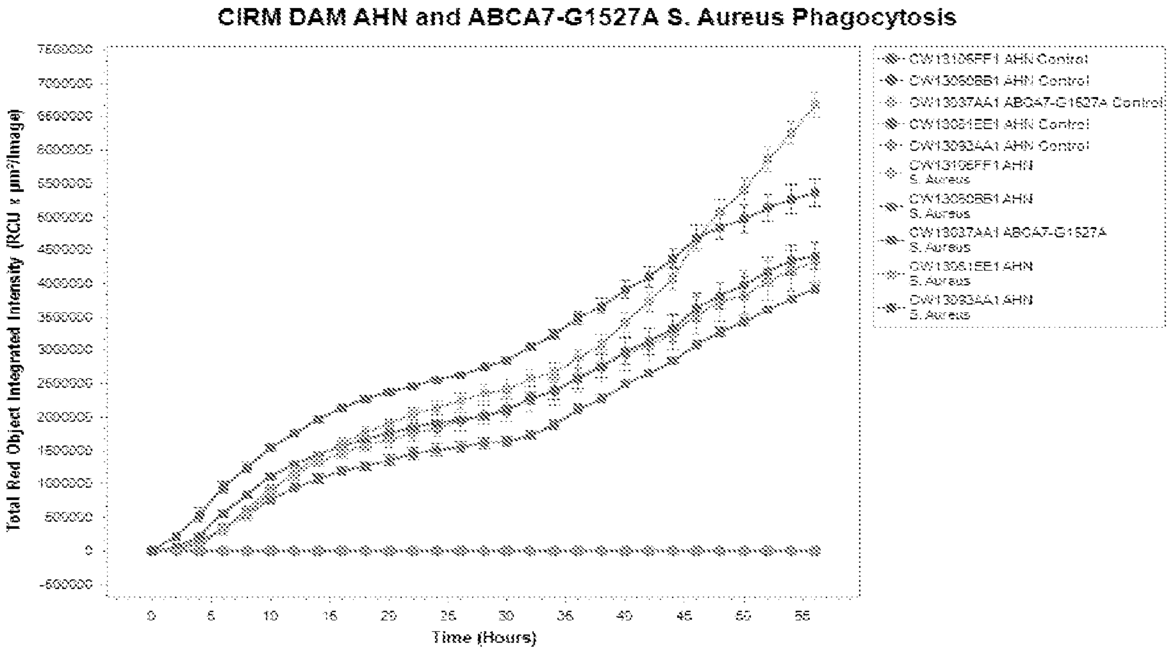

CIRM DAM AHN and ABCA7-G1527A S. Aureus Phagocytosis
Total Red Object Integrated Intensity (RCU x µm²/Image)
7500000
7000000
6500000
6000000
5500000
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
-500000
0
5
10
15
20
25
30
35
40
45
50
55
Time (Hours)
CW13106FF1 AHN Control
CW13060BB1 AHN Control
CW13037AA1 ABCA7-G1527A Control
CW13081EE1 AHN Control
CW13093AA1 AHN Control
CW13106FF1 AHN S. Aureus
CW13060BB1 AHN S. Aureus
CW13037AA1 ABCA7-G1527A S. Aureus
CW13081EE1 AHN S. Aureus
CW13093AA1 AHN S. Aureus

FIG. 47G

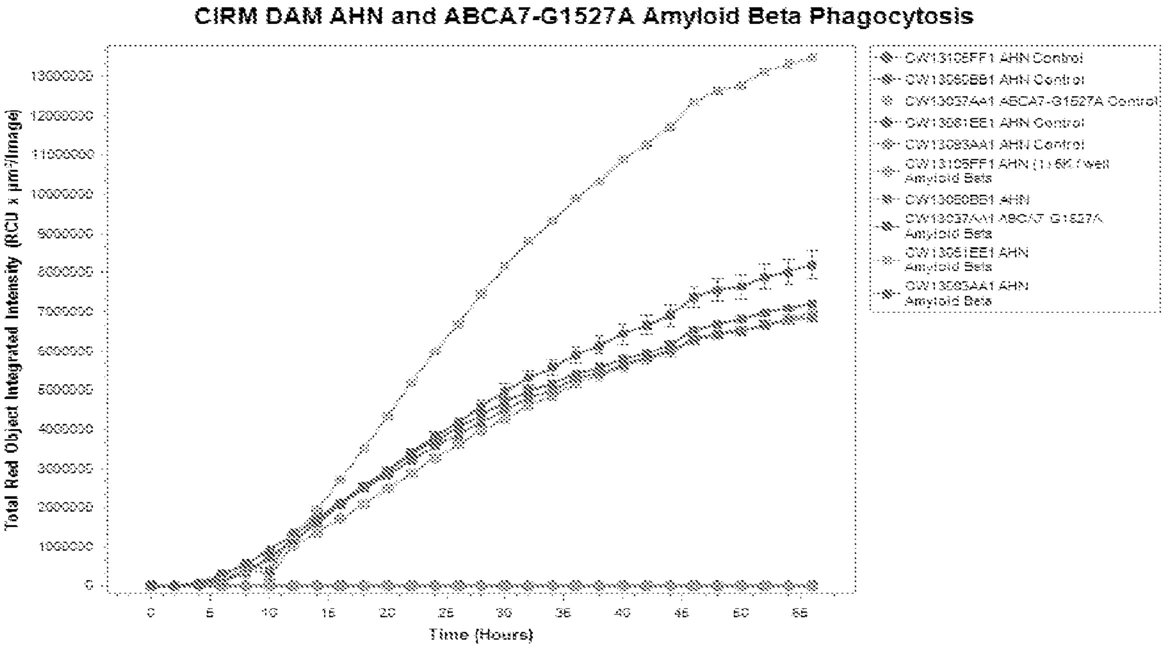


FIG. 47H

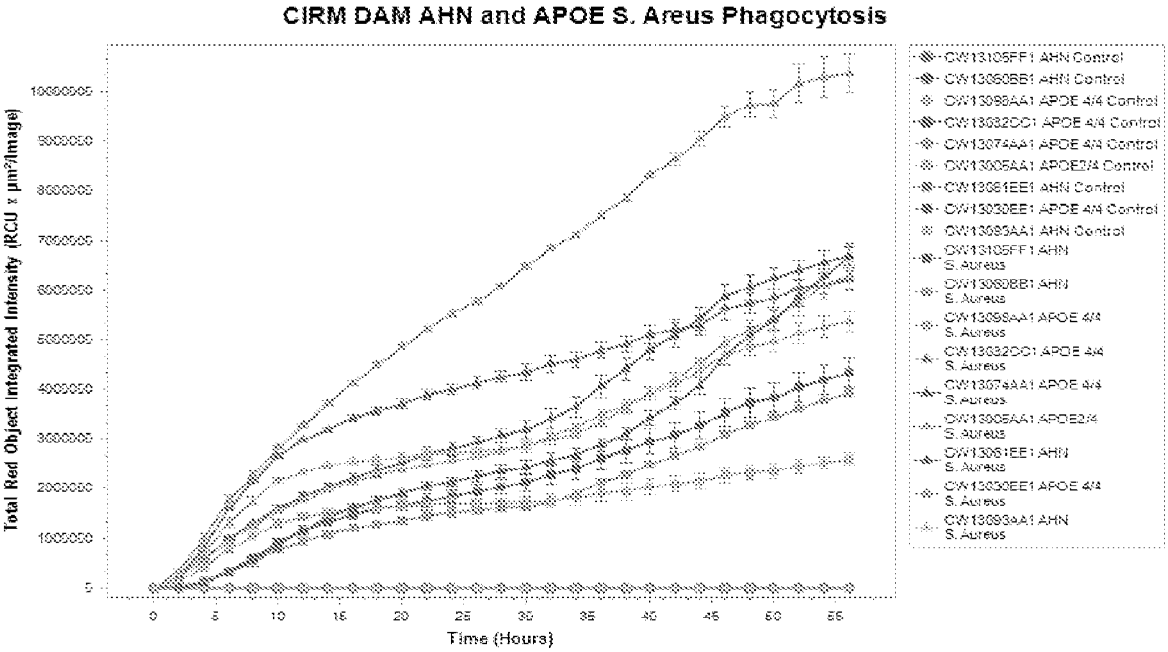

CIRM DAM AHN and APOE S. Areus Phagocytosis
Total Red Object Integrated Intensity (RCU x µm²/Image)
10000005
9000005
8000005
7000005
6000005
5000005
4000005
3000005
2000005
1000005
5
0
5
10
15
20
25
30
35
40
45
50
55
Time (Hours)
CW13105FF1 AHN Control
CW13060BB1 AHN Control
CW13098AA1 APOE 4/4 Control
CW13032CC1 APOE 4/4 Control
CW13074AA1 APOE 4/4 Control
CW13005AA1 APOE2/4 Control
CW13061EE1 AHN Control
CW13030EE1 APOE 4/4 Control
CW13093AA1 AHN Control
CW13105FF1 AHN S. Aureus
CW13060BB1 AHN S. Aureus
CW13098AA1 APOE 4/4 S. Aureus
CW13032CC1 APOE 4/4 S. Aureus
CW13074AA1 APOE 4/4 S. Aureus
CW13005AA1 APOE2/4 S. Aureus
CW13061EE1 AHN S. Aureus
CW13030EE1 APOE 4/4 S. Aureus
CW13093AA1 AHN S. Aureus

FIG. 47I

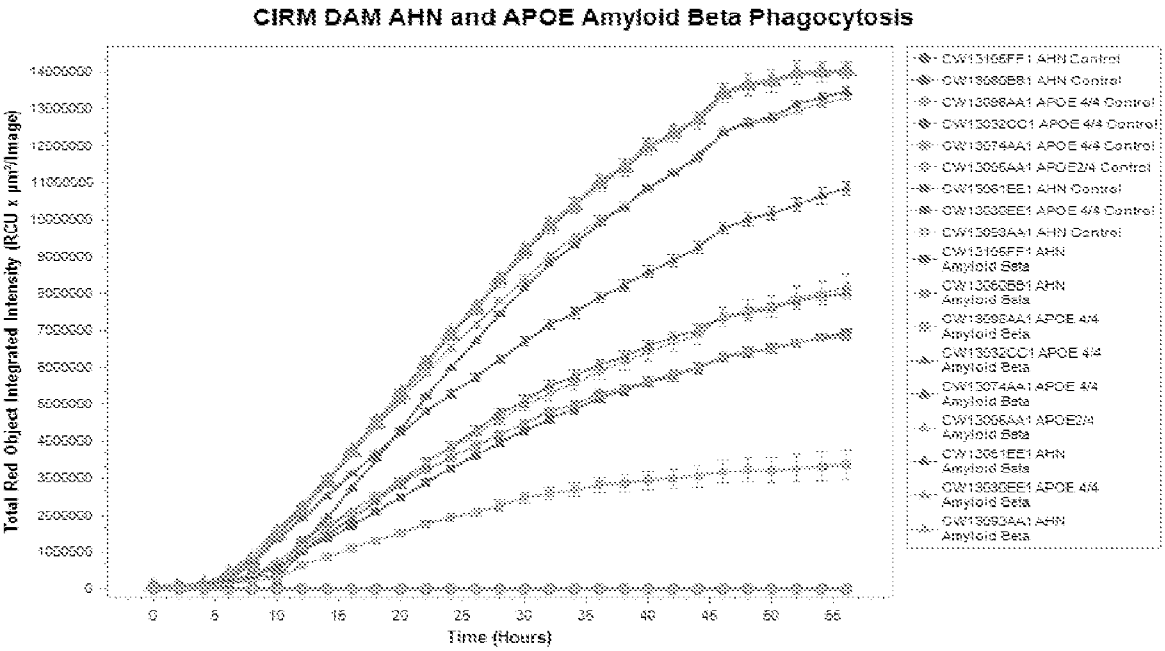
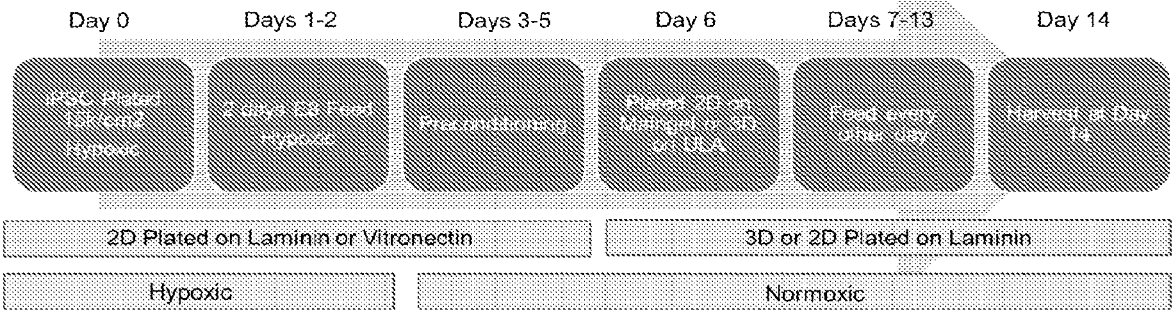

CIRM DAM AHN and APOE Amyloid Beta Phagocytosis
Total Red Object Integrated Intensity (RCU x µm²/Image)
14000000
13000000
12000000
11000000
10000000
9000000
8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
0
5
10
15
20
25
30
35
40
45
50
55
Time (Hours)
CW13105FF1 AHN Control
CW13060BB1 AHN Control
CW13098AA1 APOE 4/4 Control
CW13032CC1 APOE 4/4 Control
CW13074AA1 APOE 4/4 Control
CW13056AA1 APOE2/4 Control
CW13061EE1 AHN Control
CW13038EE1 APOE 4/4 Control
CW13053AA1 AHN Control
CW13105FF1 AHN Amyloid Beta
CW13060BB1 AHN Amyloid Beta
CW13098AA1 APOE 4/4 Amyloid Beta
CW13032CC1 APOE 4/4 Amyloid Beta
CW13074AA1 APOE 4/4 Amyloid Beta
CW13056AA1 APOE2/4 Amyloid Beta
CW13061EE1 AHN Amyloid Beta
CW13038EE1 APOE 4/4 Amyloid Beta
CW13093AA1 AHN Amyloid Beta

FIG. 47J

Generation of NPCs from iPSCs without dual SMAD Inhibition



iPSC Medium
Complete E8 Medium

Pre Conditioning Medium	
DMEM-F12	99%
CHIR	3μM
Pen/Strep	1%

NPC Differentiation Medium	
E6	98%
N2 Supplement	1%
Pen/Strep	1%

FIG. 48A

Characterization of NPCs at different days of differentiation

01279 Surface

Day of Samping	Tra161 (%)	CD56 (%)	CD15 (%)	Cell Number (1e6)
D 6	94	2	2	2
D8	43	72	2	3
D10	32	70	13	3.28
D12	9	68	18	3.64
D14	4	70	22	9.16
D16	3	66.3	18	5.34

CW50074881 Surface

Day of Sampling	Tra161 (%)	CD56 (%)	CD15 (%)	Cell Number (1e6)
D6	84	2	0	2
D8	21	86	0	5.6
D10	13	86	4	4.86
D12	6	78	9	3.94
D14	2	78	11	8.4
D16	1.2	73	10	5.48

11237.101 Surface

Day of Sampling	Tra161 (%)	CD56 (%)	CD15 (%)	Cell Number (1e6)
D6	96	2	0	2
D8	40	72	0	4.92
D10	38	75	2	5.38
D12	18	83	4	7.42
D14	3	85	12	11.08
D16	2	87	10	9.04

01279 Intracellular

Day of Sampling	SOX1	Nestin	B3 Tubulin	PAX6
D6	27	99	0	48
D8	30	99	3	95
D10	98	100	3	99
D12	71	99	5	46
D14	80	99	4	28
D16	94	99	7	49

CW50074881 Intracellular

Day of Sampling	SOX1	Nestin	B3 Tubulin	PAX6
D6	19	99	0	53
D8	38	100	7	94
D10	99	100	2	99
D12	67	99	4	45
D14	60	99	5	27
D16	98	99	10	49

11237.101 Intracellular

Day of Sampling	SOX1	Nestin	B3 Tubulin	PAX6
D6	13	99	0	73
D8	35	100	5	96
D10	93	100	1	100
D12	58	99	1	32
D14	73	99	5	21
D18	99	99	12	42

FIG. 48B

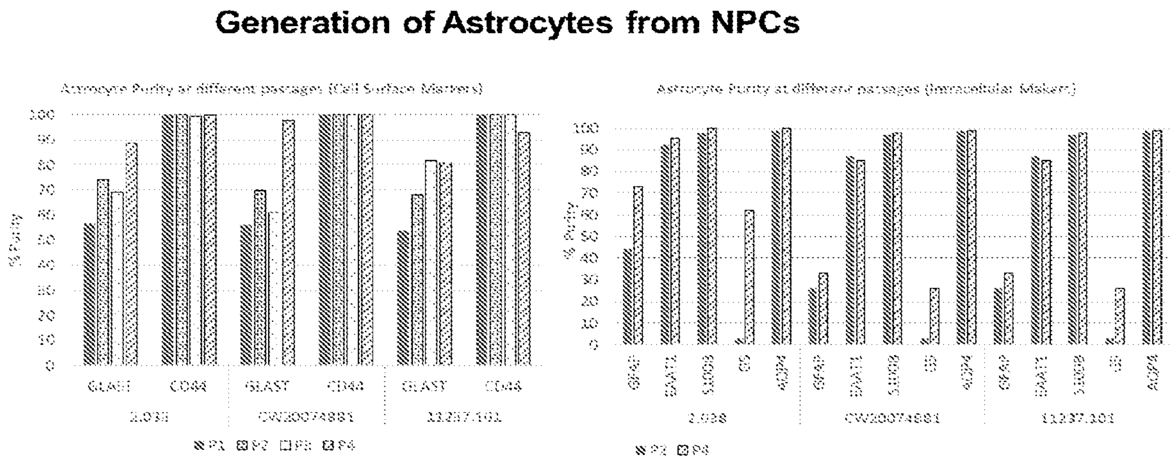


FIG. 48C

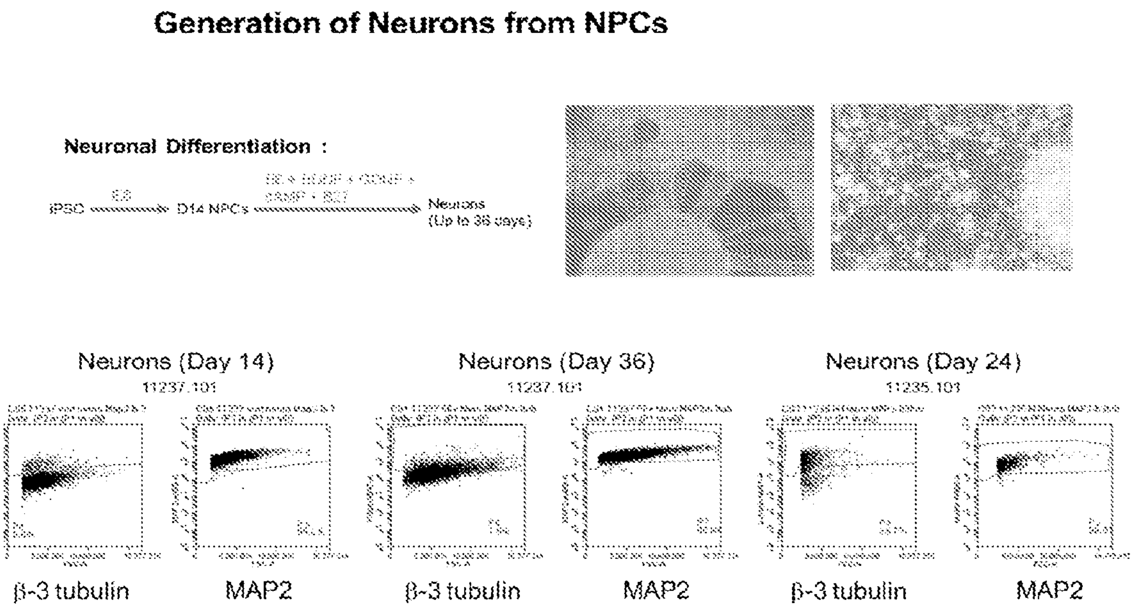


FIG. 48D

Summary of iPSC-Derived Cell Types and Surfaces for Derivation	
Surface	**iPSC-Derived Cell Lineages**
Carboxyl	Endothelial Cells
Amine	Hematopoietic Progenitor Cells (HPC)
Amine	Mesenchymal Stem Cells (MSC)
Carboxyl, Amine, Primaria, Non Tissue Culture, Ultra Low Attachment, Tissue Culture	Microglia
Amine, Primaria, Carboxyl	Brain Microvascular Endothelial Cells (BMEC)
Laminin, Ultra Low Attachment	Neural Progenitor Cells (NPC)
Amine (Stage 1), Tissue Culture (Stage 2)	Pericytes

FIG. 49

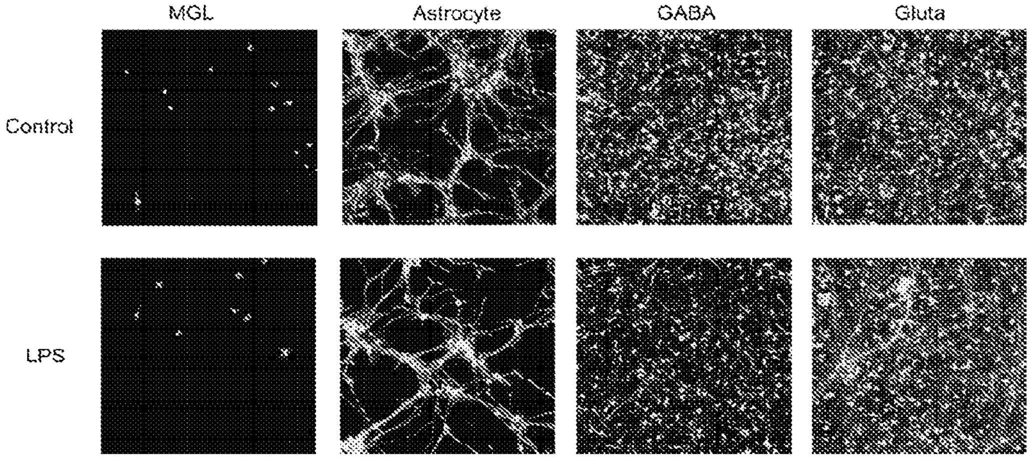


FIG. 50A

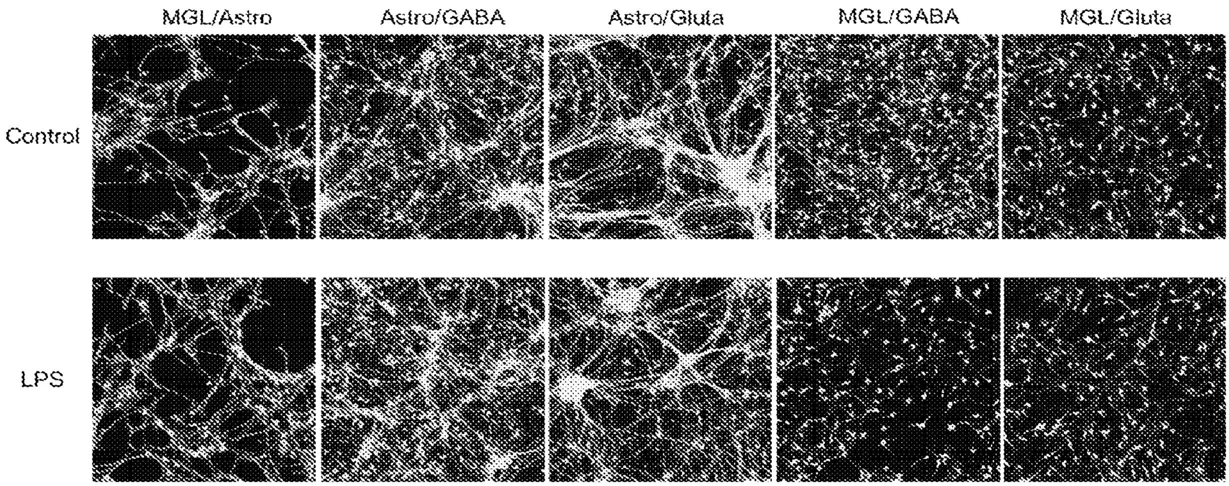


FIG. 50B

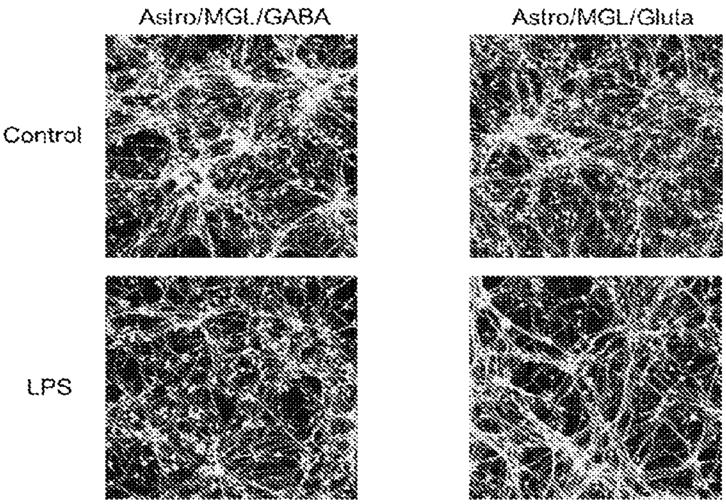


FIG. 50C

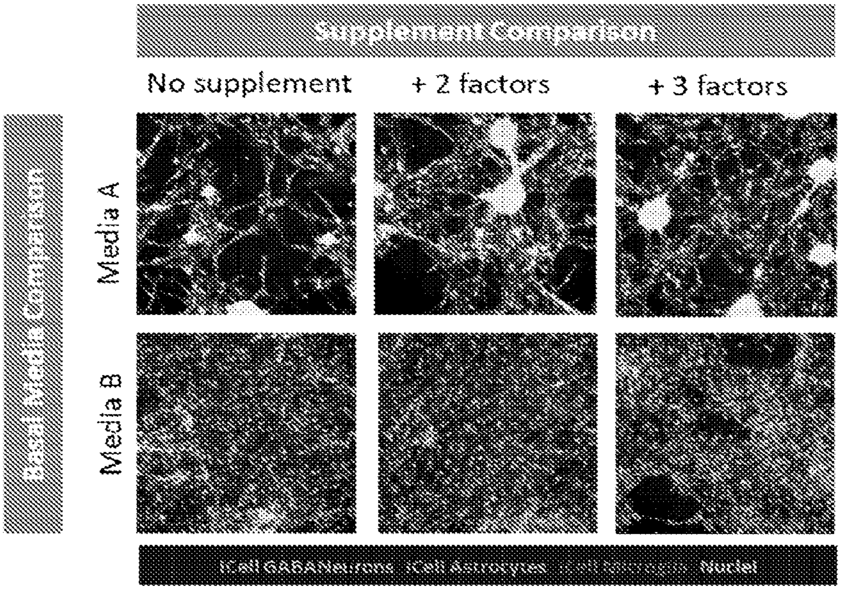


FIG. 50D

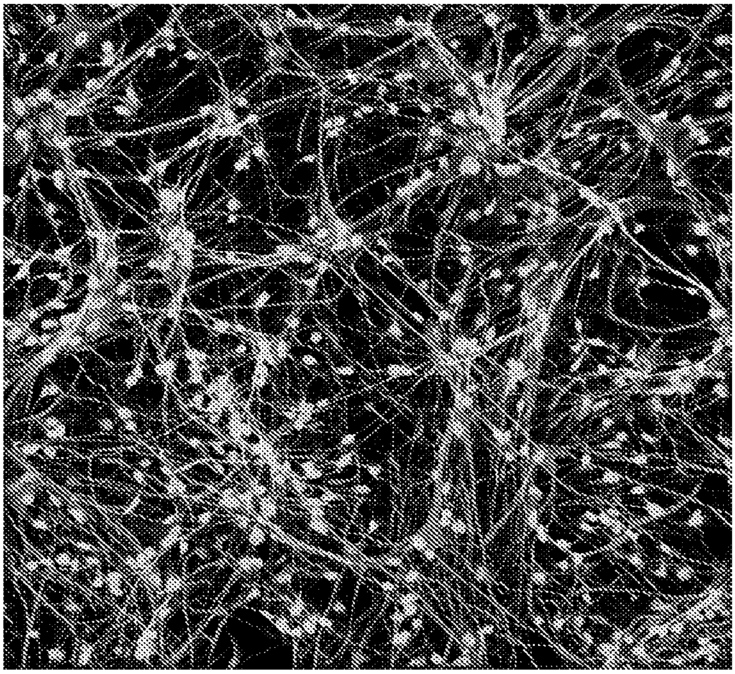


FIG. 51

Cell Conditions:

1 MGL (mono-culture)
2 GABAneurons mono-culture
3 Astrocytes mono-culture
4 GABA + Astrocytes co-culture
5 MGL + GABA co-culture
6 MGL + Astrocytes co-culture
7 HO + GABA co-culture
8 MGL + GABA + Astrocytes

Stimulation Conditions:

1 Unstimulated
2 LPS
3 INF-gamma
4 LPS + INF-gamma

Follow Tri-culture Application Protocol:

Stimulate on Day 14
Collect 150 uL per well Day 15 (24 hr)
Collect 150 uL per well Day 16 (48 hr)
Fix plates after 48 hr collection for ICC

Luminex Analytes:

TNF-alpha, IL-6, IL-21, IL-3, IL-8, IL-10, CCL2, VEGF, beta-NGF, IFN-gamma, CCL3, CCL4, CCL5, IL-1 alpha, FAS ligand, IL-17, IL-2, GMCSF, FGF-basic, IL-1 beta, Granzyme B, ICAM-1, IL1-RII, CXCL11, GCSF, TNF-RI, IL-12, CX3CL1, IL-4, CXCL9, C3, C1Q

FIG. 52

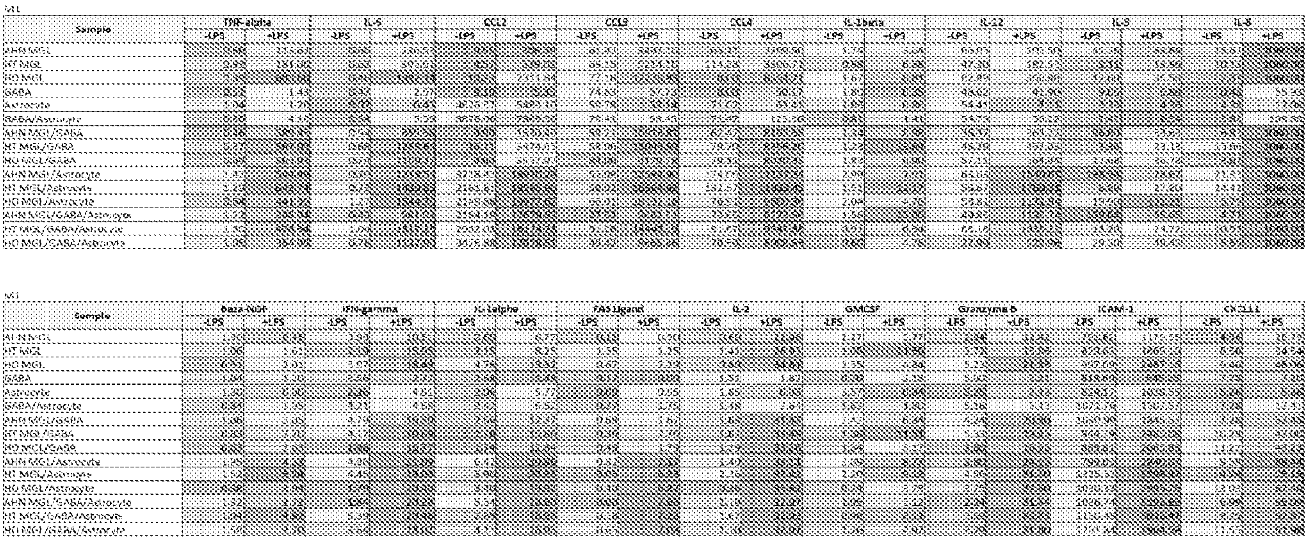
Signature of Reactive Astrocyte (A1)

Sample	CX3CL1	
	-LPS	+LPS
AHN MGL	447.30	421.57
HT MGL	563.46	1448.49
HO MGL	628.27	1111.71
GABA	160.59	179.05
Astrocyte	934.57	28.10
GABA/Astrocyte	526.03	815.25
AHN MGL/GABA	365.70	1170.05
HT MGL/GABA	344.39	1110.16
HO MGL/GABA	647.31	776.76
AHN MGL/Astrocyte	285.37	1628.66
HT MGL/Astrocyte	255.51	999.61
HO MGL/Astrocyte	23.70	1760.47
AHN MGL/GABA/Astrocyte	459.49	1061.53
HT MGL/GABA/Astrocyte	191.85	1187.40
HO MGL/GABA/Astrocyte	238.68	1207.98

Signature of Non-Reactive Astrocyte (A2)

Sample	FGF-basic	
	-LPS	+LPS
AHN MGL	0.27	0.62
HT MGL	0.19	0.77
HO MGL	0.56	0.89
GABA	0.24	0.16
Astrocyte	0.50	0.27
GABA/Astrocyte	0.32	0.47
AHN MGL/GABA	0.27	0.95
HT MGL/GABA	0.38	1.23
HO MGL/GABA	0.10	0.76
AHN MGL/Astrocyte	0.26	1.13
HT MGL/Astrocyte	0.27	1.06
HO MGL/Astrocyte	0.31	1.03
AHN MGL/GABA/Astrocyte	0.27	0.92
HT MGL/GABA/Astrocyte	0.40	0.93
HO MGL/GABA/Astrocyte	0.42	0.98

FIG. 53



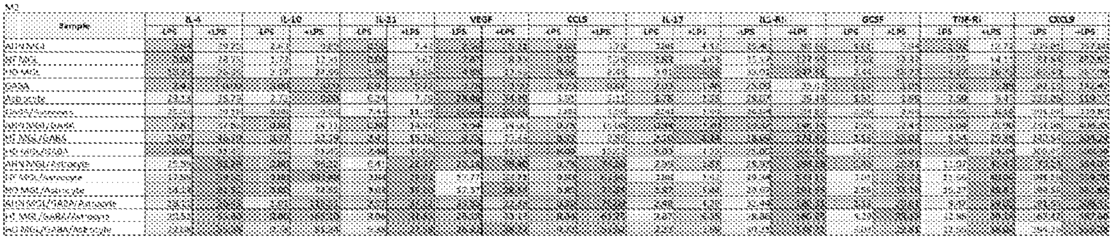
M1

Sample	TNF-alpha		IL-6		CCL2		CCL3		CCL4		IL-1beta		IL-22		IL-5		IL-8	
	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS
AHN MGL	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HT MGL	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HO MGL	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
GABA	[illegible]	1.43	[illegible]	2.57	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
GABA/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
AHN MGL/GABA	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HT MGL/GABA	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HO MGL/GABA	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
AHN MGL/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HT MGL/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HO MGL/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
AHN MGL/GABA/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HT MGL/GABA/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HO MGL/GABA/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

M1

Sample	Beta-NGF		IFN-gamma		IL-1alpha		FAS Ligand		IL-2		GMCSF		Granzyme B		ICAM-1		CXCL11	
	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS
AHN MGL	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HT MGL	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HO MGL	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
GABA	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
GABA/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
AHN MGL/GABA	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HT MGL/GABA	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HO MGL/GABA	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
AHN MGL/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HT MGL/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HO MGL/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
AHN MGL/GABA/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HT MGL/GABA/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HO MGL/GABA/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

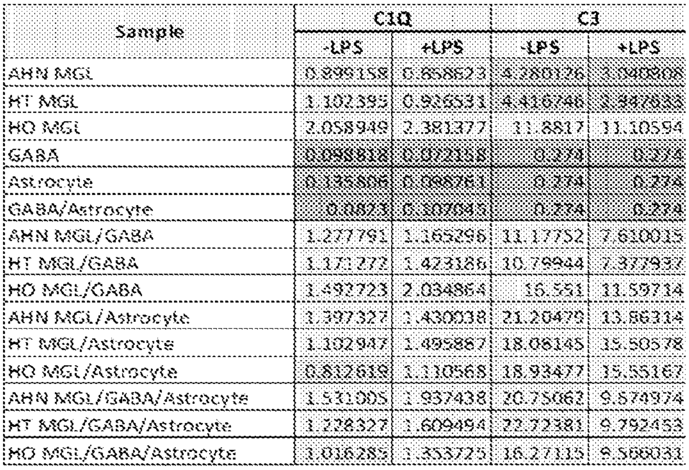
FIG. 54



M2

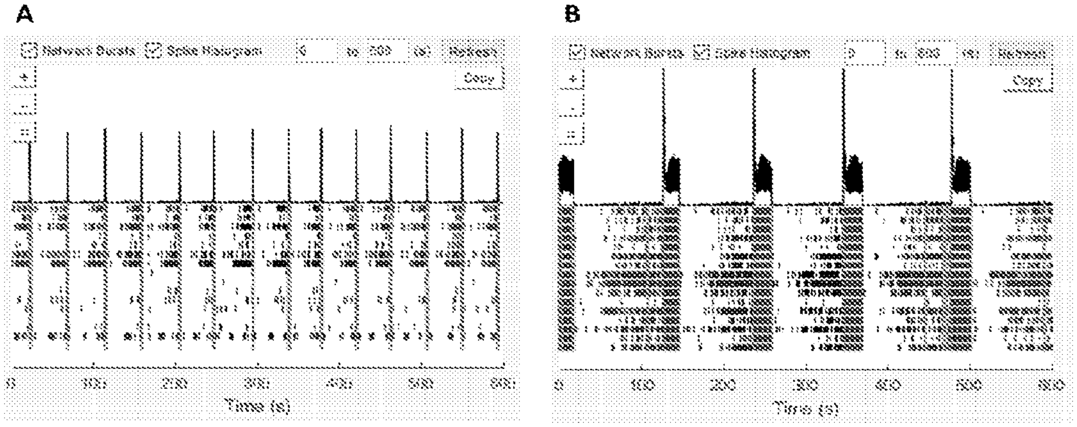
Sample	IL-4		IL-10		IL-21		VEGF		CCL5		IL-17		IL-RI		GCSF		TNF-RI		CXCL9	
	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS
AHN MGL	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HT MGL	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HO MGL	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
GABA	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
GABA/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
AHN MGL/GABA	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HT MGL/GABA	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HO MGL/GABA	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
AHN MGL/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HT MGL/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HO MGL/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
AHN MGL/GABA/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HT MGL/GABA/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
HO MGL/GABA/Astrocyte	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

FIG. 55



Sample	C1Q		C3	
	-LPS	+LPS	-LPS	+LPS
AHN MGL	0.899158	0.858623	4.280126	3.040808
HT MGL	1.102395	0.926531	4.416746	3.947633
HO MGL	2.058949	2.381377	11.8817	11.10594
GABA	0.098818	0.072158	0.274	0.274
Astrocyte	0.135806	0.098761	0.274	0.274
GABA/Astrocyte	0.0823	0.107045	0.274	0.274
AHN MGL/GABA	1.277791	1.165296	11.17752	7.610015
HT MGL/GABA	1.171272	1.423186	10.79944	7.377937
HO MGL/GABA	1.492723	2.034864	16.551	11.59714
AHN MGL/Astrocyte	1.397327	1.430038	21.20479	13.86314
HT MGL/Astrocyte	1.102947	1.495887	18.08145	15.50578
HO MGL/Astrocyte	0.812619	1.110568	18.93477	15.55167
AHN MGL/GABA/Astrocyte	1.531005	1.937438	20.75062	9.574974
HT MGL/GABA/Astrocyte	1.228327	1.609494	22.72381	9.792453
HO MGL/GABA/Astrocyte	1.016285	1.353725	16.27115	9.566033

FIG. 56



A. GlutaNeurons and Astrocytes on MEA.
Raster plot of synchronous network bursting.

B. Addition of Microglia start to remodel neural networks.

FIGS. 57A-57B

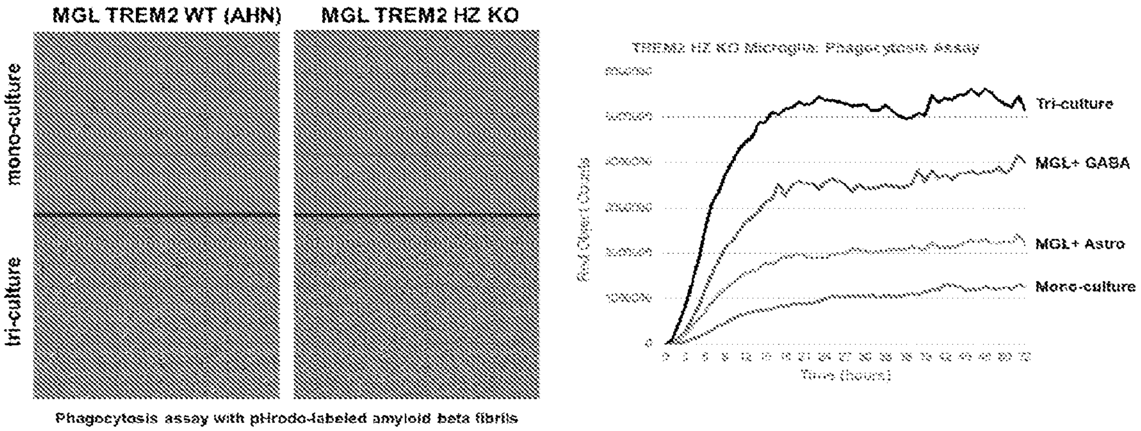

MGL TREM2 WT (AHN)
MGL TREM2 HZ KO
mono-culture
tri-culture
Phagocytosis assay with pHrodo-labeled amyloid beta fibrils
TREM2 HZ KO Microglia: Phagocytosis Assay
600000
500000
400000
300000
200000
100000
0
Red Object Counts
Time (hours)
Tri-culture
MGL+ GABA
MGL+ Astro
Mono-culture

FIG. 58

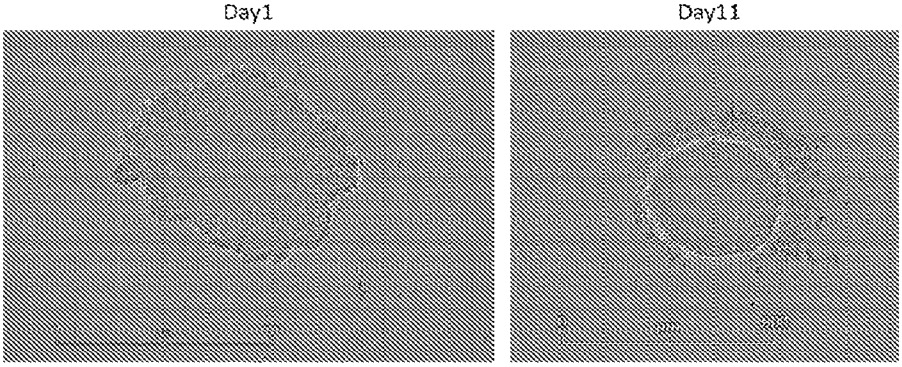

Day1
Day11

FIG. 59

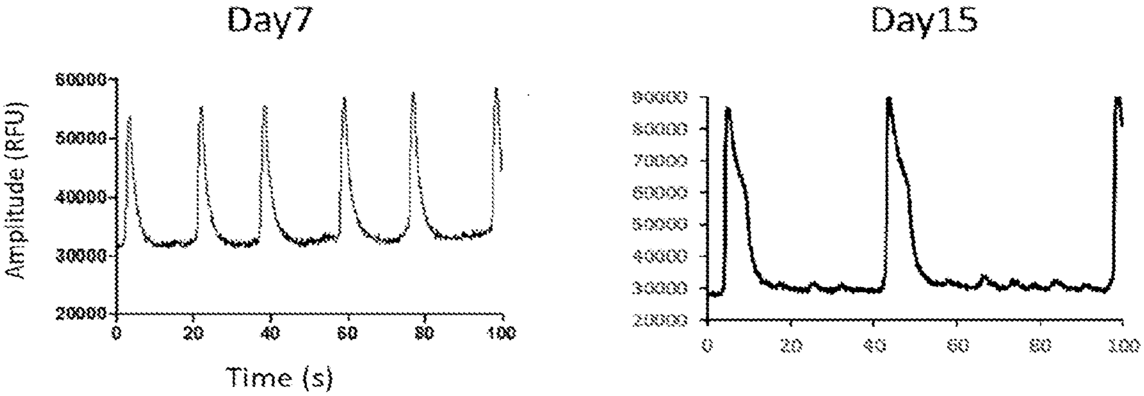

Day7
Amplitude (RFU)
60000
50000
40000
30000
20000
0
20
40
60
80
100
Time (s)
Day15
90000
80000
70000
60000
50000
40000
30000
20000
0
20
40
60
80
100

FIG. 60

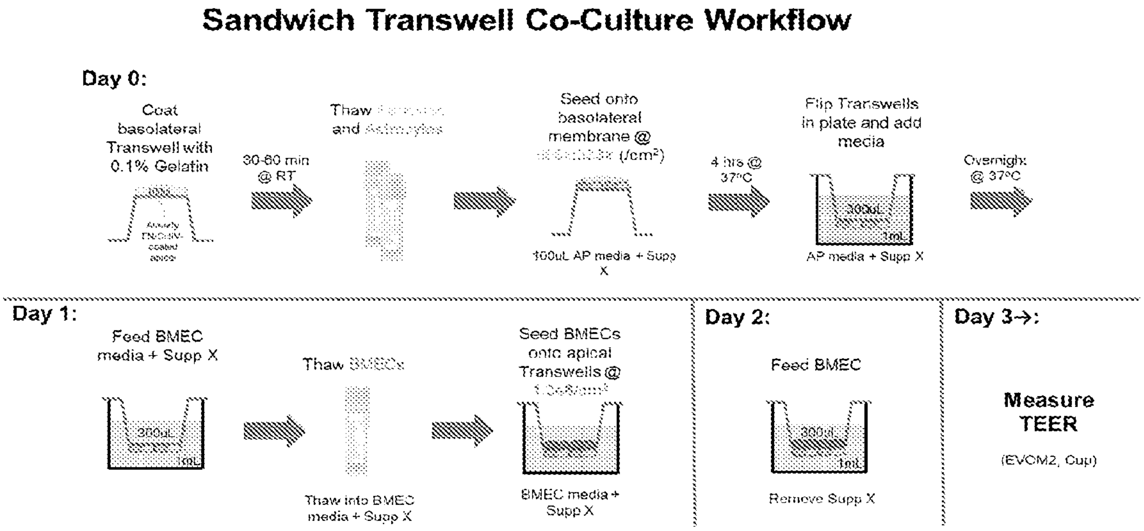

Sandwich Transwell Co-Culture Workflow
Day 0:
Coat basolateral Transwell with 0.1% Gelatin
100uL
Already FN:Col IV-coated apical
30-60 min @ RT
Thaw Pericytes and Astrocytes
Seed onto basolateral membrane @ (/cm²)
100uL AP media + Supp X
4 hrs @ 37°C
Flip Transwells in plate and add media
300uL
1mL
AP media + Supp X
Overnight @ 37°C
Day 1:
Feed BMEC media + Supp X
300uL
1mL
Thaw BMECs
Thaw into BMEC media + Supp X
Seed BMECs onto apical Transwells @
BMEC media + Supp X
Day 2:
Feed BMEC
300uL
1mL
Remove Supp X
Day 3→:
Measure TEER
(EVOM2, Cup)

FIG. 61

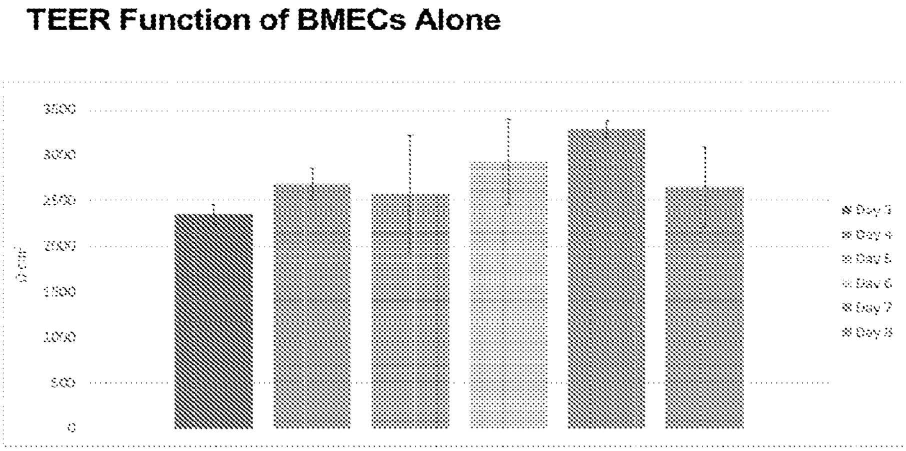

TEER Function of BMECs Alone
3500
3000
2500
2000
1500
1000
500
0
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8

FIG. 62

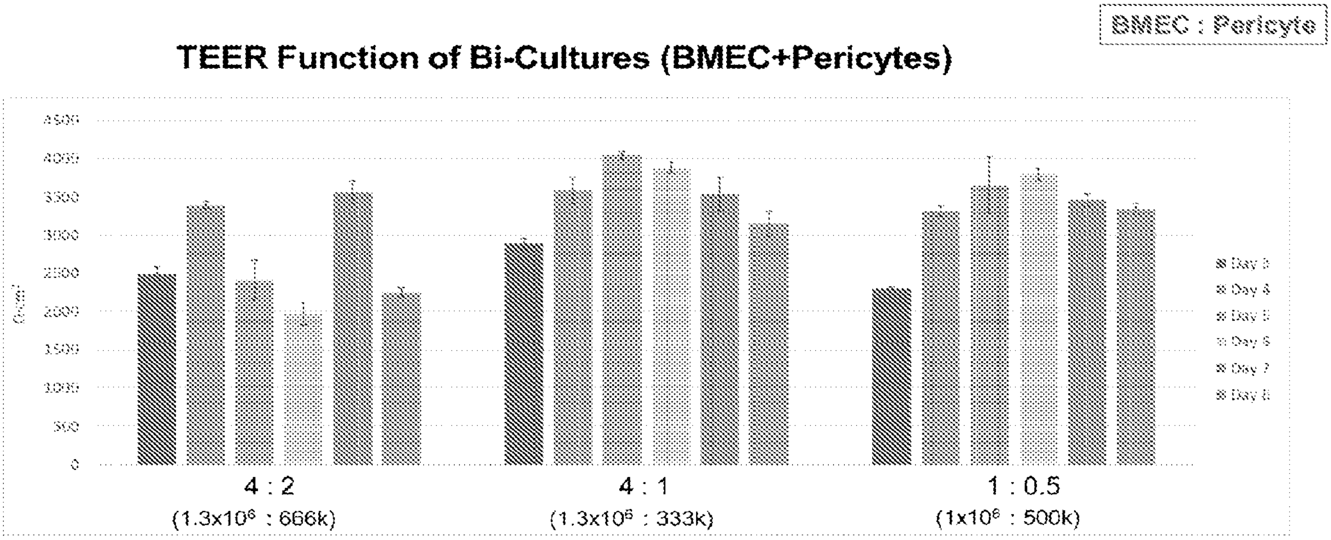

BMEC : Pericyte
TEER Function of Bi-Cultures (BMEC+Pericytes)
Ω·cm²
4500
4000
3500
3000
2500
2000
1500
1000
500
0
4 : 2
(1.3x10⁶ : 666k)
4 : 1
(1.3x10⁶ : 333k)
1 : 0.5
(1x10⁶ : 500k)
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8

FIG. 63

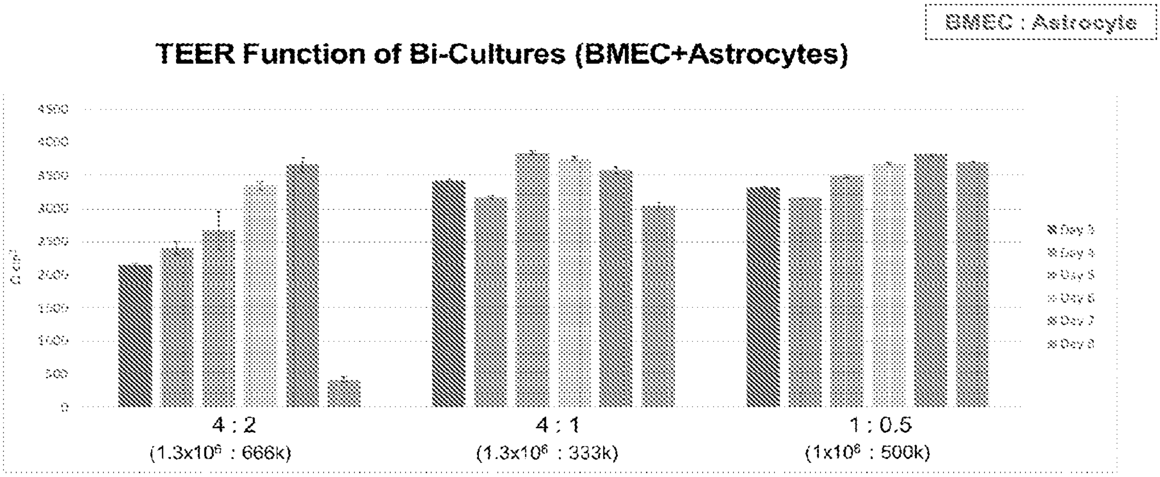

BMEC : Astrocyte
TEER Function of Bi-Cultures (BMEC+Astrocytes)
Ω·cm²
4500
4000
3500
3000
2500
2000
1500
1000
500
0
4 : 2
(1.3x10⁶ : 666k)
4 : 1
(1.3x10⁶ : 333k)
1 : 0.5
(1x10⁶ : 500k)
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8

FIG. 64

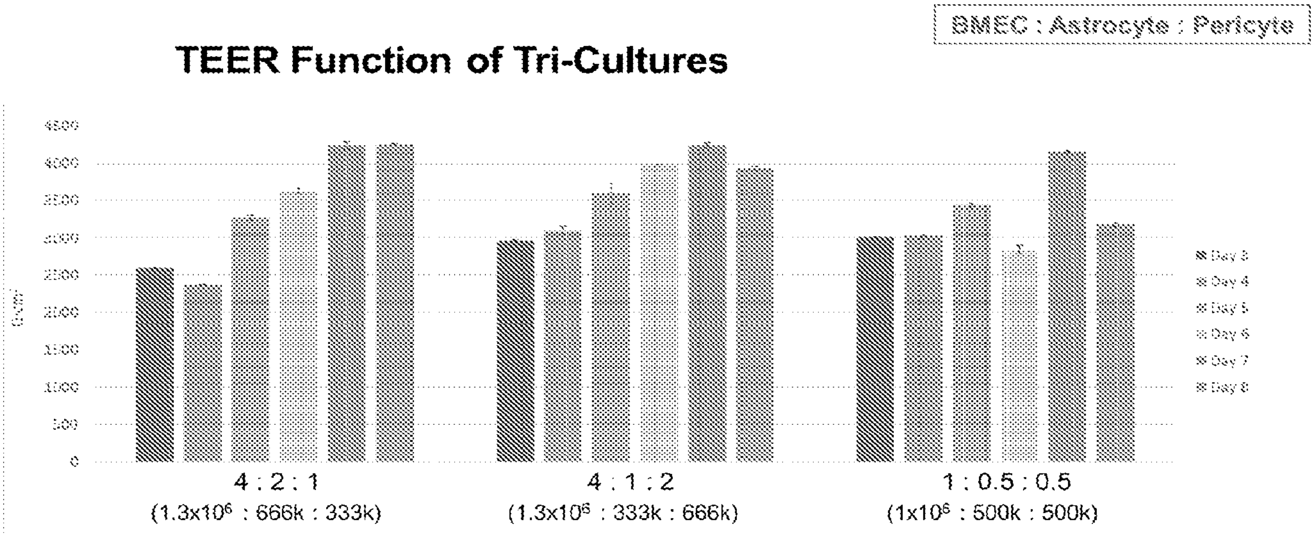

BMEC : Astrocyte : Pericyte
TEER Function of Tri-Cultures
Ohm
4500
4000
3500
3000
2500
2000
1500
1000
500
0
4 : 2 : 1
(1.3x10^6 : 666k : 333k)
4 : 1 : 2
(1.3x10^6 : 333k : 666k)
1 : 0.5 : 0.5
(1x10^6 : 500k : 500k)
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8

FIG. 65

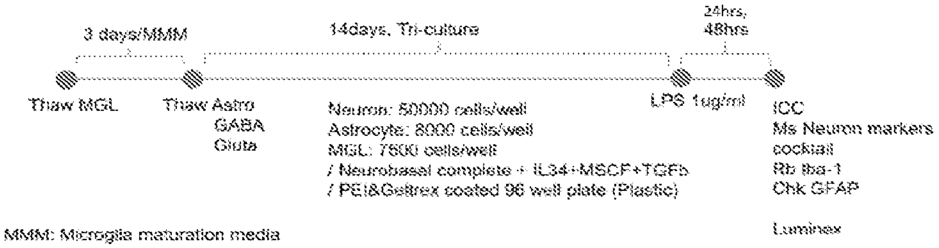

3 days/MMM
14days, Tri-culture
24hrs,
48hrs
Thaw MGL
Thaw Astro
GABA
Gluta
Neuron: 50000 cells/well
Astrocyte: 8000 cells/well
MGL: 7600 cells/well
/ Neurobasal complete + IL34+MSCF+TGFb
/ PEI&Geltrex coated 96 well plate (Plastic)
LPS 1ug/ml
ICC
Ms Neuron markers
cocktail
Rb Iba-1
Chk GFAP
Luminex
MMM: Microglia maturation media

FIG. 66

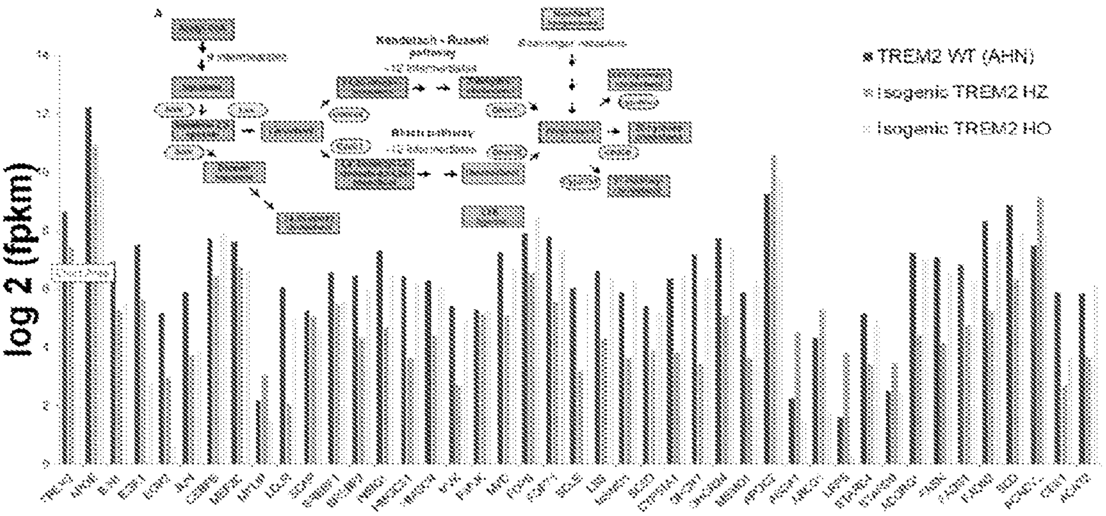

log 2 (fpkm)
Kandutsch - Russell pathway
Bloch pathway
Scavenger receptors
TREM2 WT (AHN)
Isogenic TREM2 HZ
Isogenic TREM2 HO
TREM2
APOE
SCD

FIG. 67

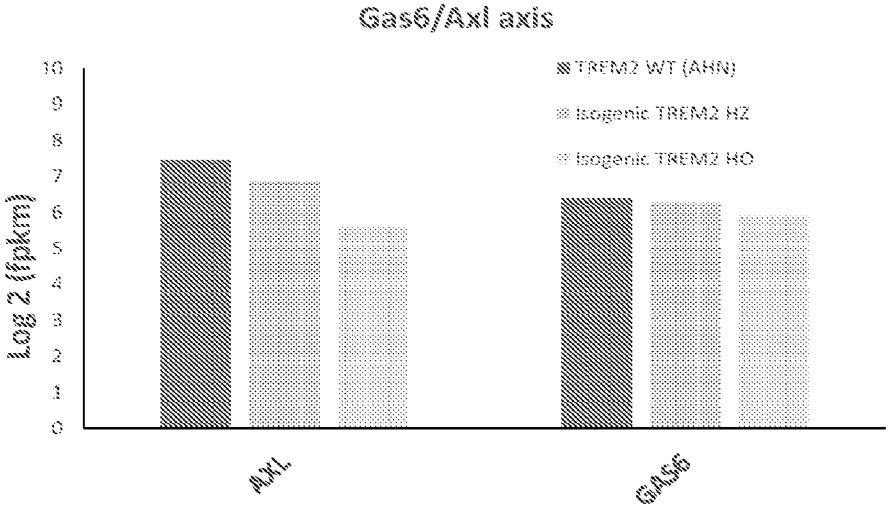

Gas6/Axl axis
Log 2 (fpkm)
10
9
8
7
6
5
4
3
2
1
0
TREM2 WT (AHN)
Isogenic TREM2 HZ
Isogenic TREM2 HO
AXL
GAS6

FIG. 68

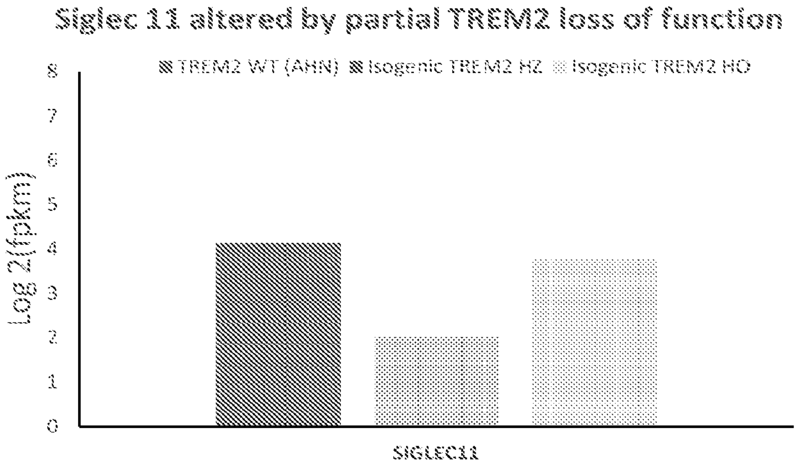


FIG. 69

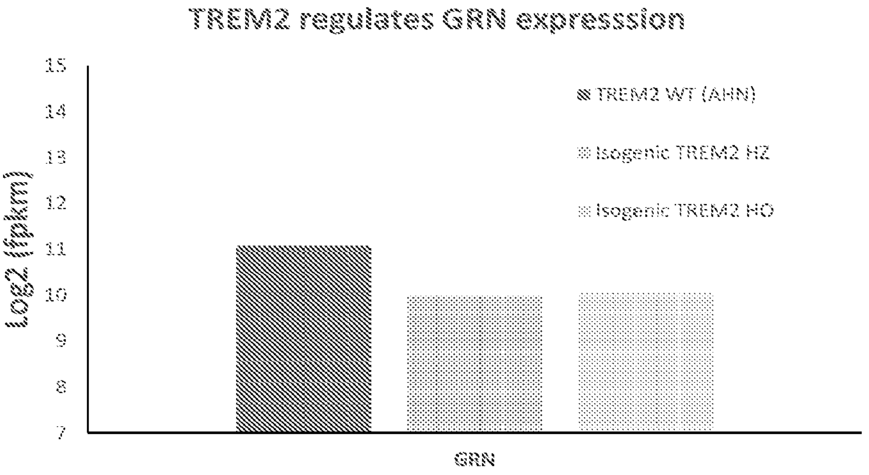


FIG. 70A

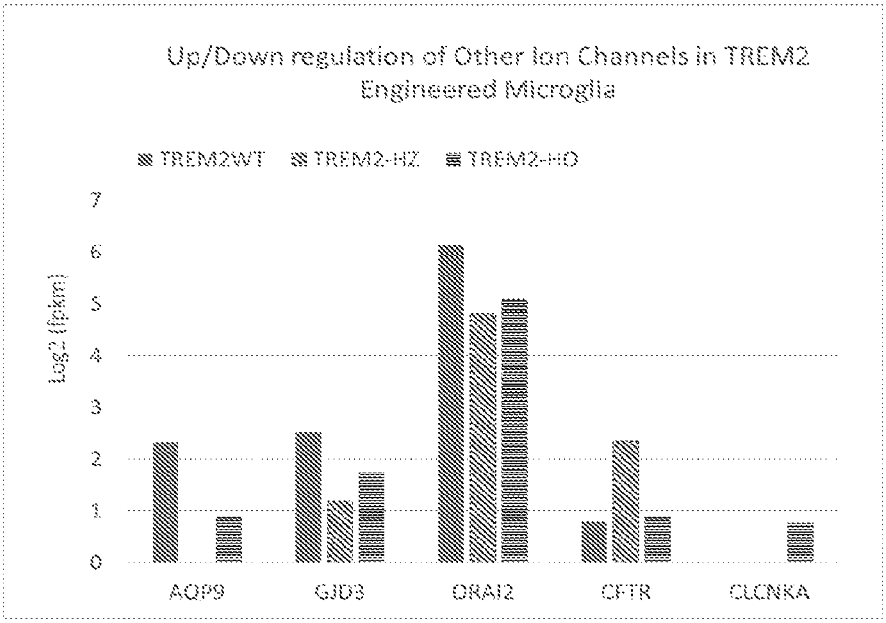


FIG. 70B

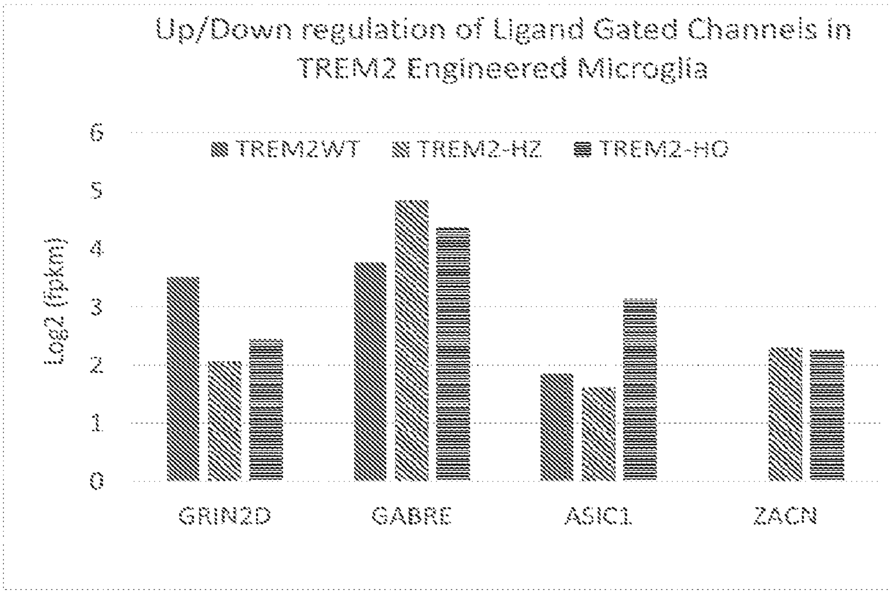


FIG. 70C

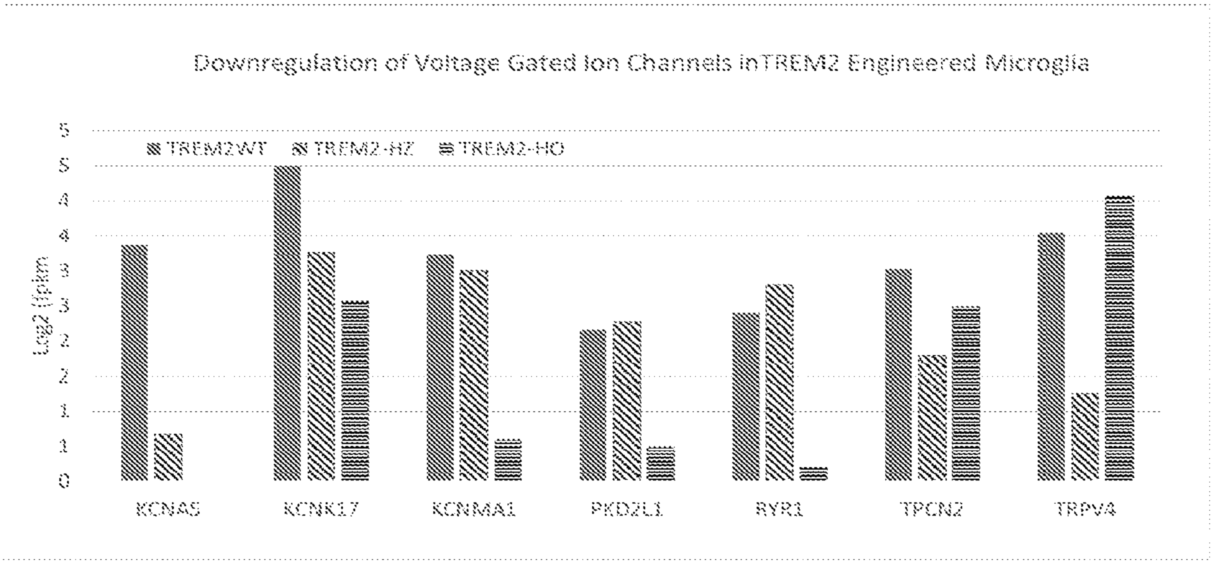


FIG. 70D

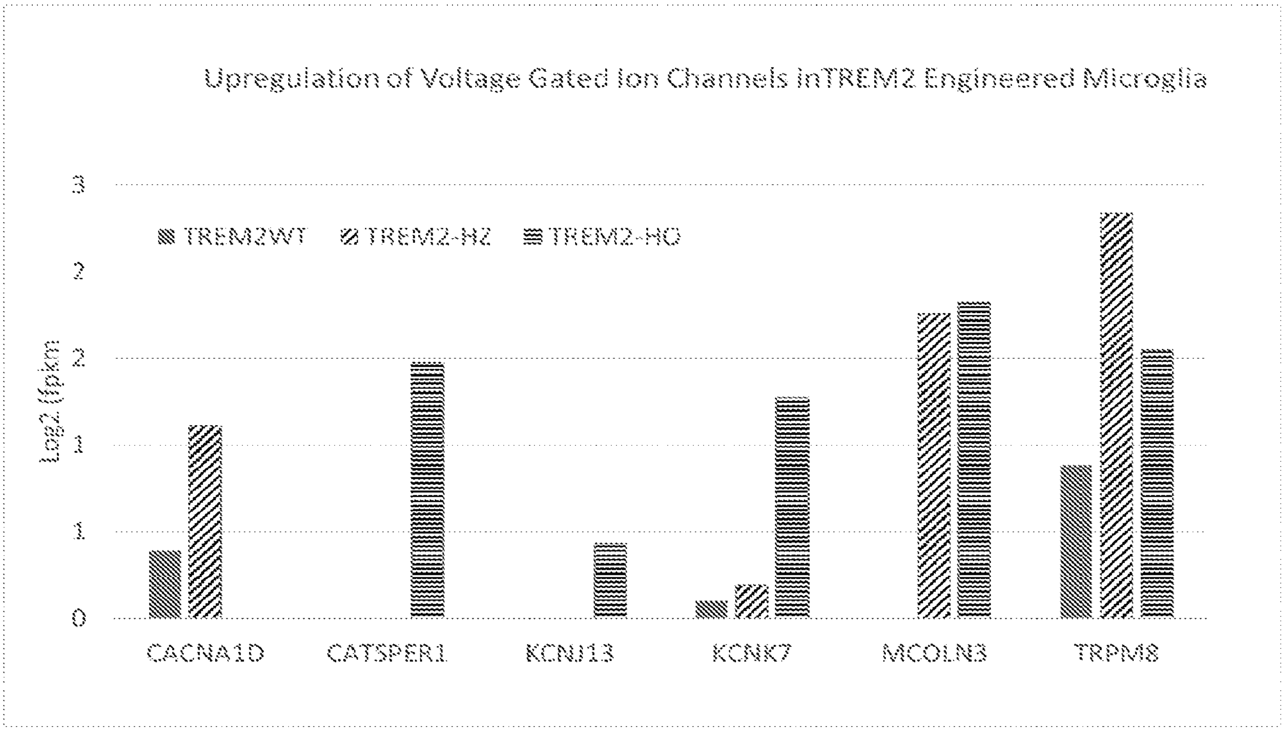


FIG. 70E

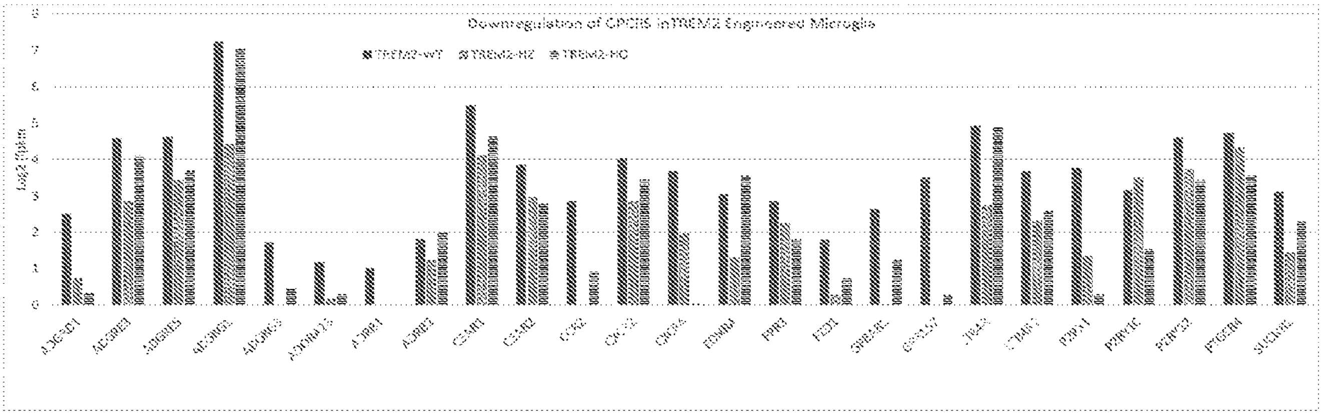


FIG. 71A

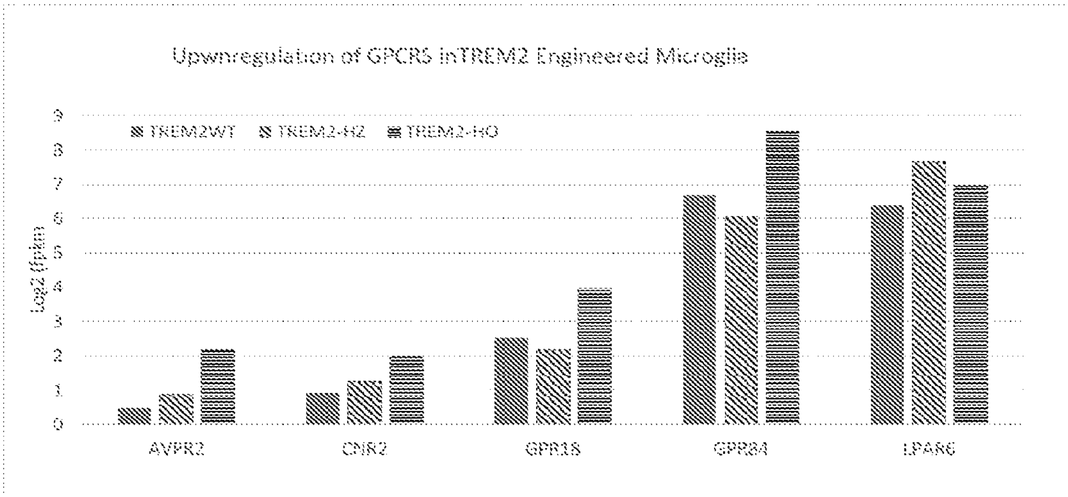


FIG. 71B

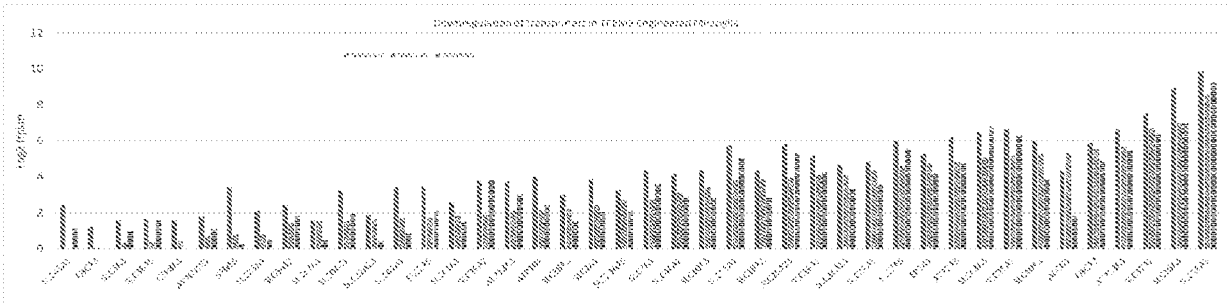


FIG. 72A

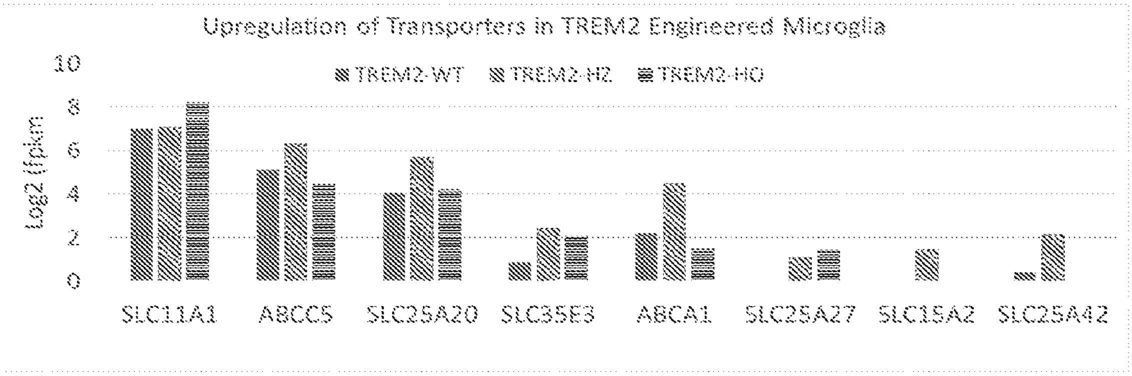


FIG. 72B

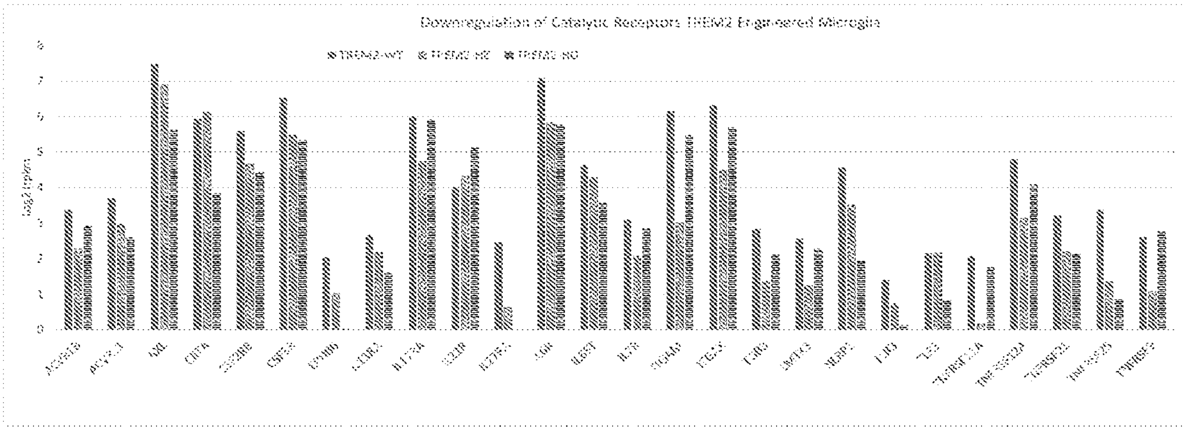


FIG. 73A

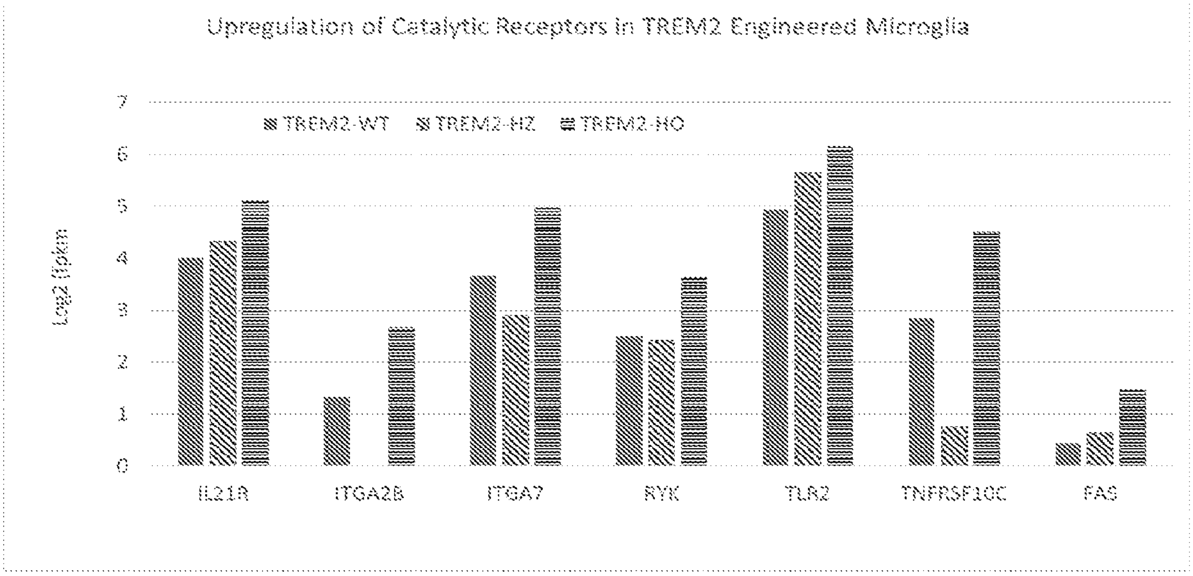


FIG. 73B

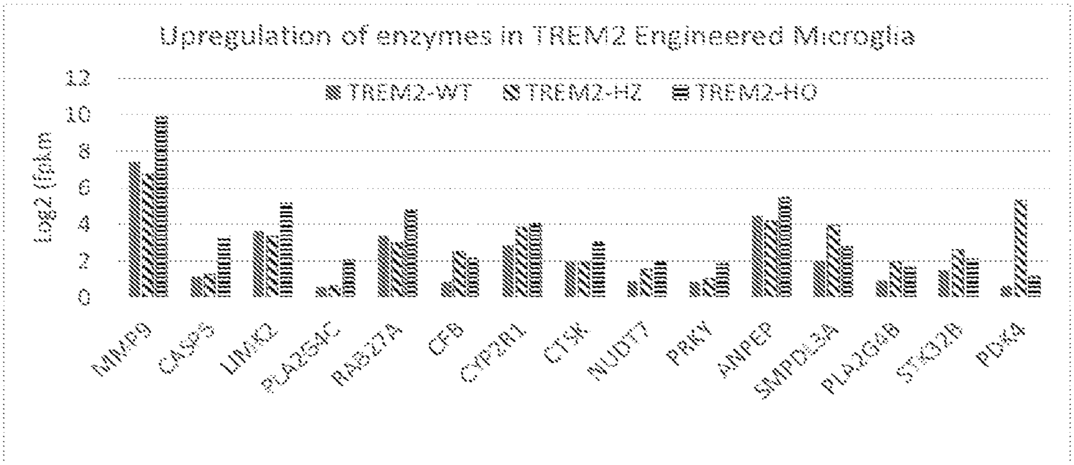


FIG. 74A

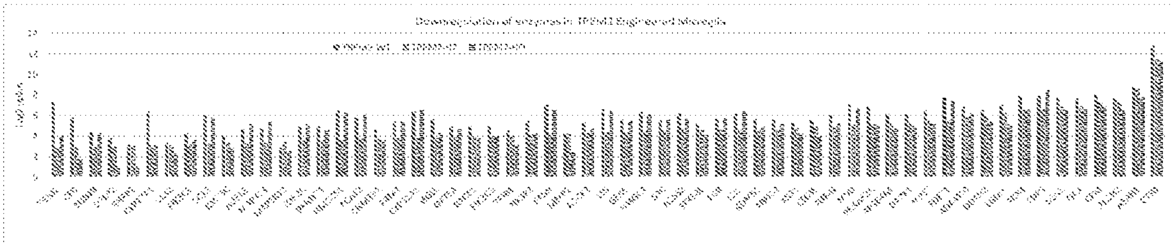


FIG. 74B

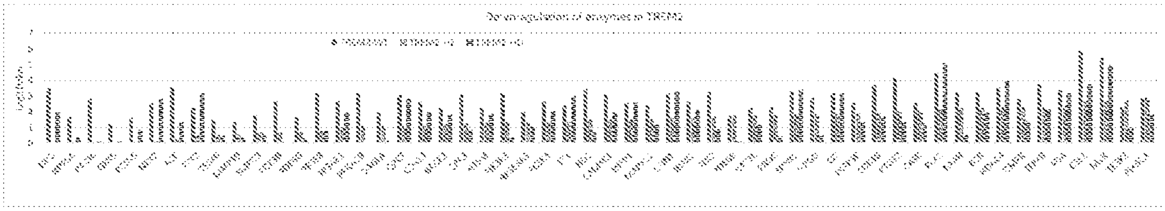


FIG. 74C

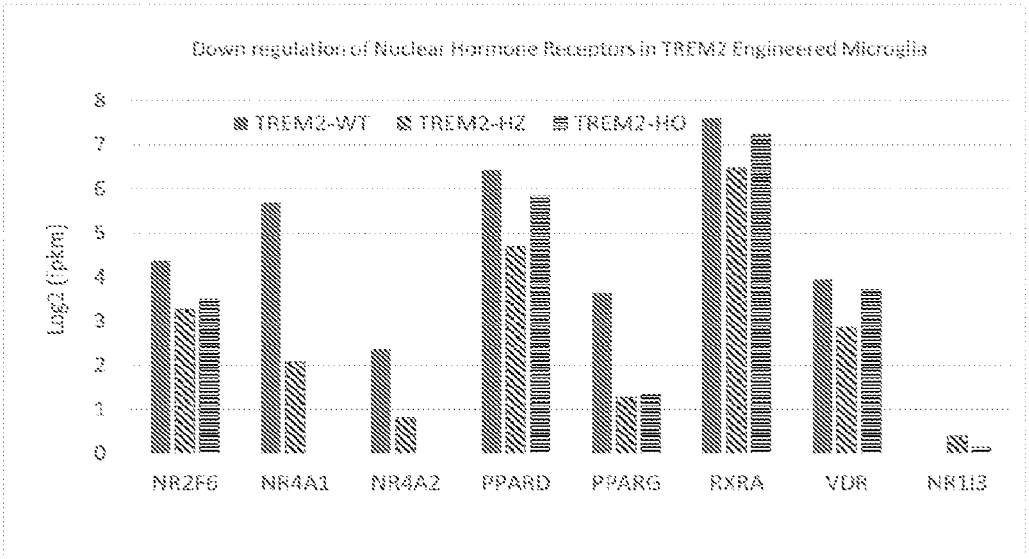


FIG. 75

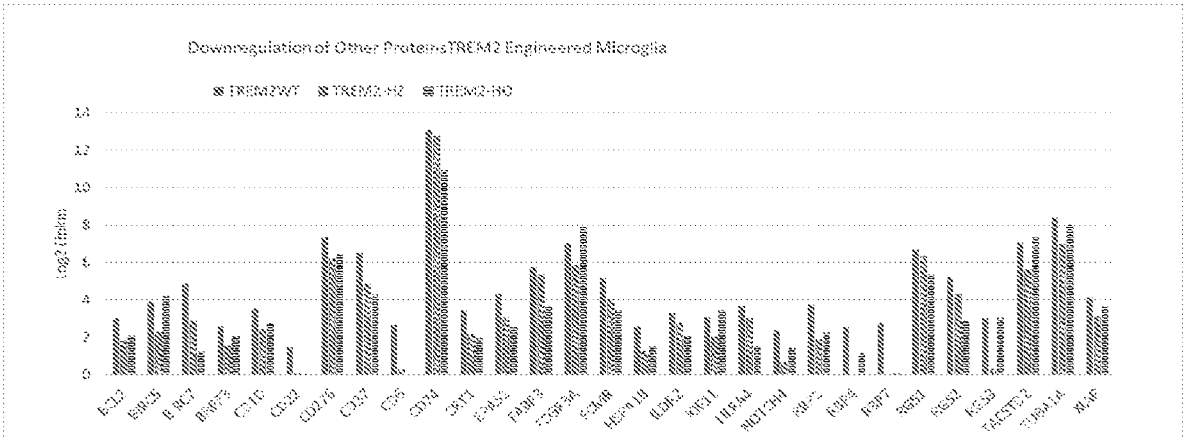


FIG. 76A

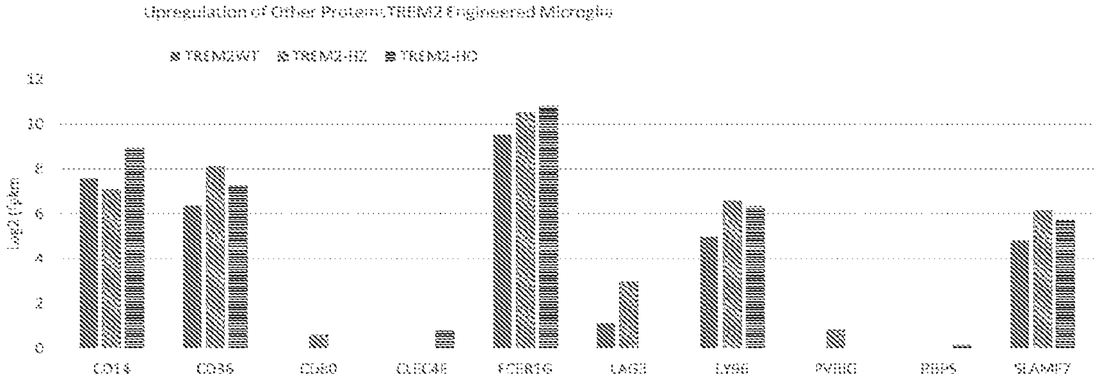


FIG. 76B

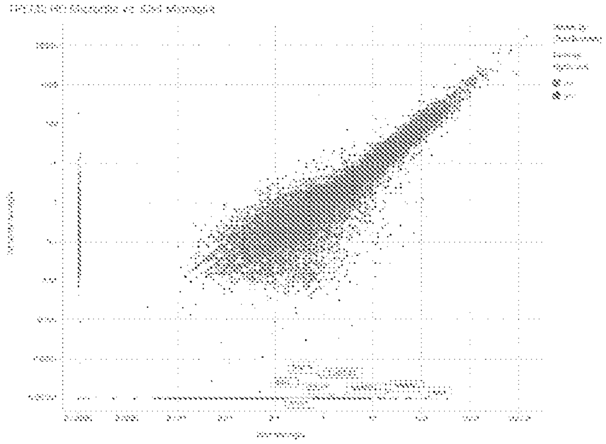


FIG. 77A

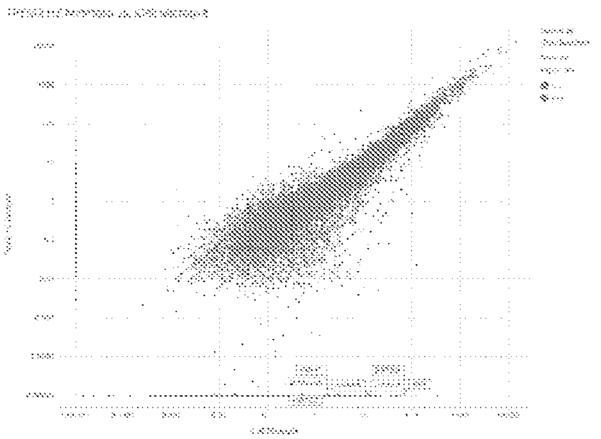


FIG. 77B

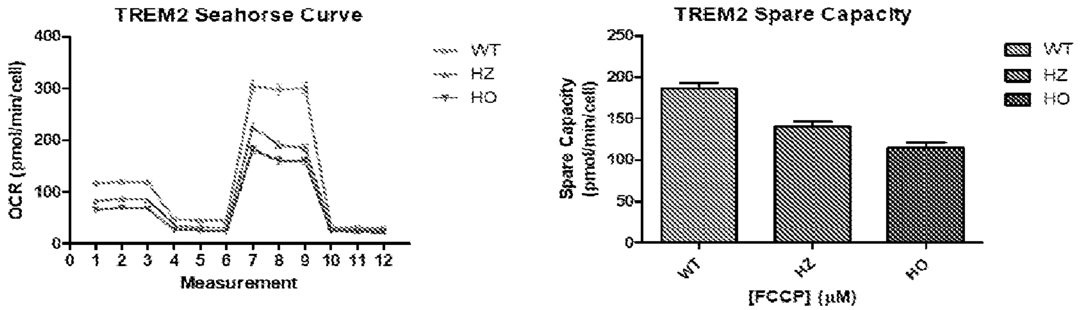


FIG. 78

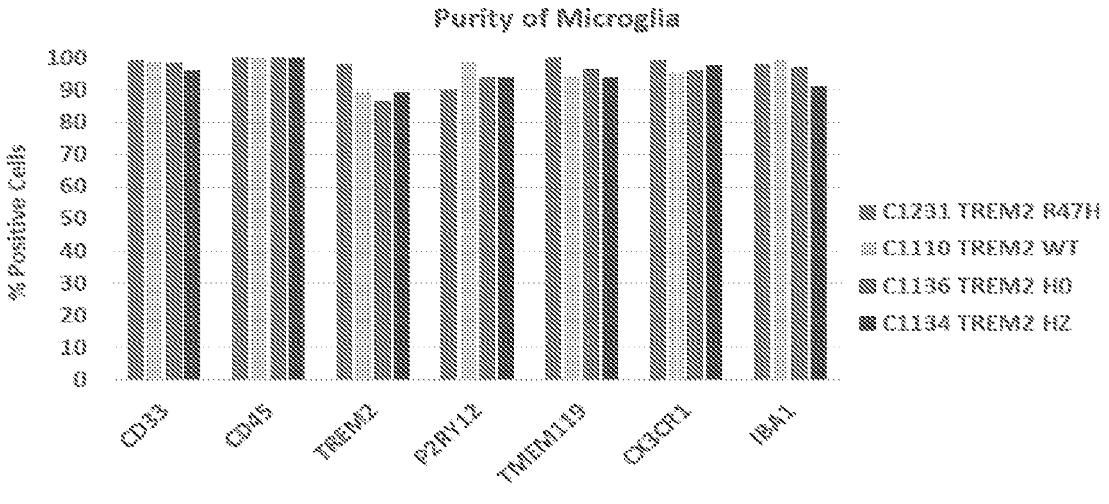


FIG. 79A

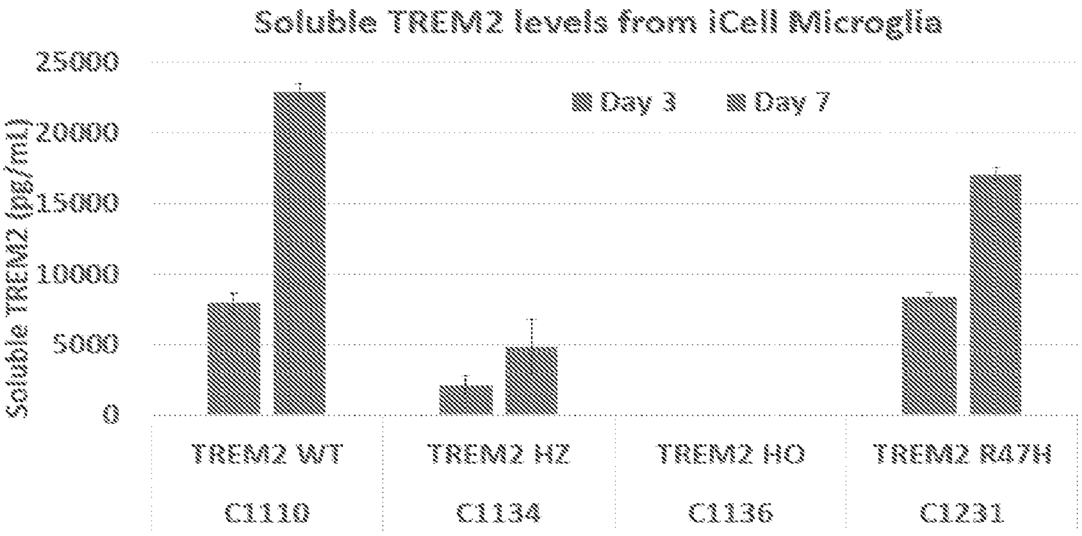


FIG. 79B

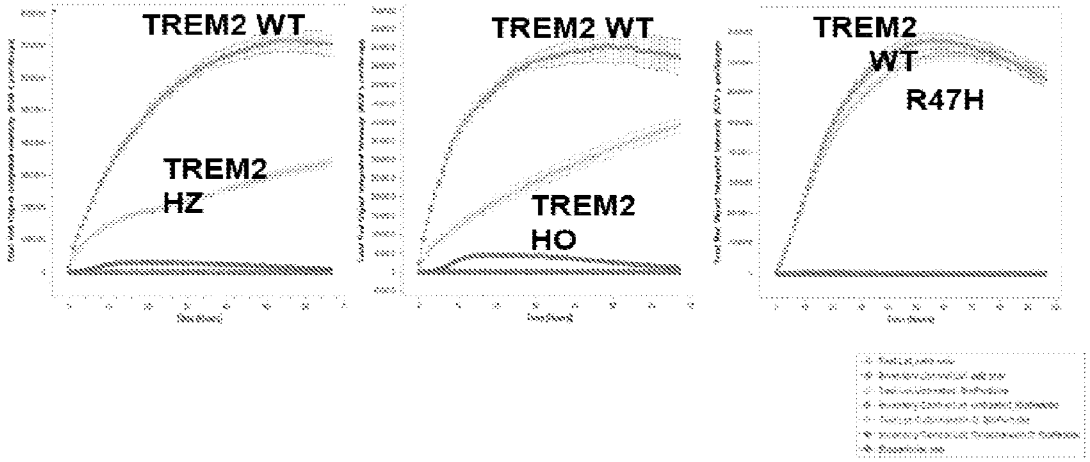


FIG. 79C

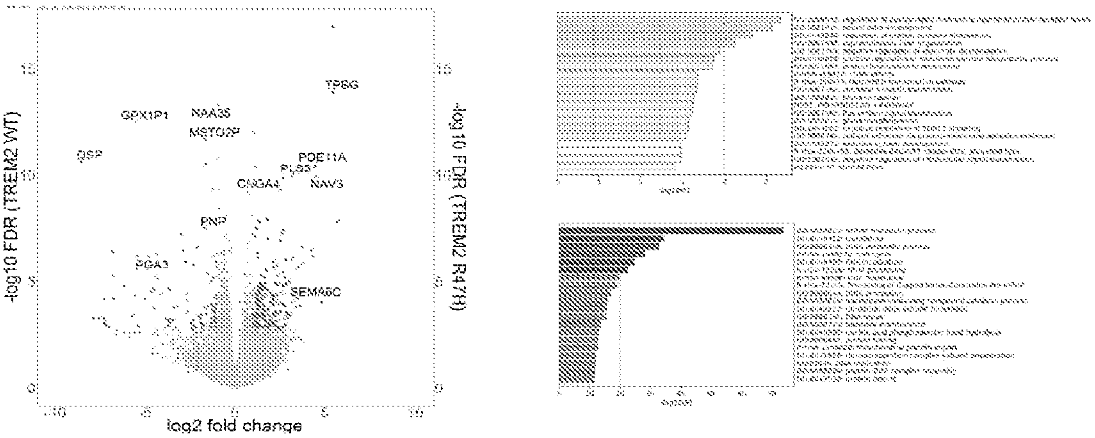


FIG. 80A

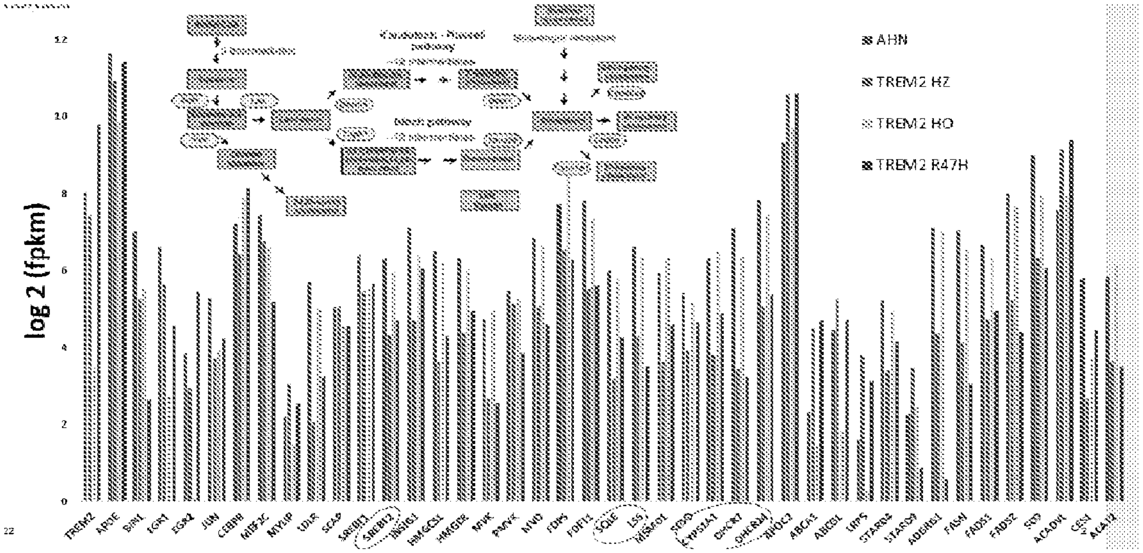

log 2 (fpkm)
0
2
4
6
8
10
12
AHN
TREM2 HZ
TREM2 HO
TREM2 R47H
TREM2
APOE
BIN1
EGR1
EGR2
JUN
CEBPB
MEF2C
MYLIP
LDLR
SCAP
SREBF1
SREBF2
INSIG1
HMGCS1
HMGCR
MVK
PMVK
MVD
FDPS
FDFT1
SQLE
LSS
MSMO1
SC5D
CYP51A1
DHCR7
DHCR24
APOC2
ABCA1
ABCG1
LRP5
STARD4
STARD9
ADGRB1
FASN
FADS1
FADS2
SCD
ACADVL
CES1
ACAT1

FIG. 80B